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## C-C CKR-1, C-C CHEMOKINE RECEPTOR

This invention relates to the field of cytokine receptors, their antibodies, and their use as diagnostic and therapeutic agents.

BACKGROUND OF THE INVENTION

Cytokines are biological molecules which affect inflammatory and immune-related effector cells. The inflammatory cytokines, or "chemokines," have a variety of biological properties including selective leukocyte chemotaxis and activation. These chemokines form a superfamily, denoted in the literature alternatively as the PF4 superfamily or the intercrines, that has been divided into two classes based on whether the first two conserved cysteine residues are separated by an intervening amino acid (C-X-C, or  $\alpha$ ), or whether they are adjacent (C-C, or  $\beta$ ). The C-X-C class members include, for example, interleukin-8 (IL-8), melanocyte growth stimulating factor (MGSA), and platelet factor 4 (PF4), while the C-C class includes RANTES (Regulated on Activation, Normal T Expressed and Secreted) and monocyte chemotactic peptide-1 (MCP-1). The C-X-C class exerts proinflammatory activity mainly through their action on neutrophils, whereas the C-C class appears to be monocyte chemoattractants.

Much attention has been focused on receptor/ligand interactions in this superfamily, with more data being available on C-X-C than C-C chemokine binding. It has become clear that chemokine receptor/ligand interactions on target inflammatory cells seem to be strictly regulated. For example, no cross competition for binding sites has been observed on either monocytes or neutrophils between members of the C-X-C or C-C branches (Leonard, E. J. et al. Immunol. Today 11:97-101, 1990; Samanta, A. K. et al. J. Exp. Med. 169:1185-1189, 1989; Yoshimura, T. et al. J. Immunol. 145:292-7, 1990), consistent with the differential chemoattractant effects on these two cell types. Direct binding data for the C-C chemokines is surprisingly sparse.

Human MCP-1 has been reported to bind to monocytes with an affinity of about 2 nM, with no sites detectable on neutrophils (Valente, A. J. et al. Biochem. Biophys. Res. Commun. 176:309-14, 1991; Yoshimura, T. et al. J. Immunol. 145:292-7, 1990). A single report shows human Act-2, a human MIP-1 $\beta$  (HuMIP-1 $\beta$ ) variant, binding to between 7,000 and 45,000 sites on PBMC with an affinity of between 7.8 and 12 nM (Napolitano, M. et al. J. Exp. Med. 172:285-289, 1990). Kwon and colleagues have characterized the binding of murine MIP-1 $\alpha$  on a mouse T cell and a macrophage cell line, finding a  $K_d$  of 1.5 and 0.9 nM, respectively (Oh, K. O. et al. J. Immunol. 147:2978-83, 1991).

Most of the molecular details regarding leukocyte motility remain to be elucidated. Recently, however, the receptors for the anaphylatoxin C5a (Gerard, N. P. et al. Nature 349:614-7, 1991), the bacterial formylated tripeptide fMLP (Boulay, F. et al. Biochemistry 29:11123-11133, 1990), and the C-X-C chemokine IL-8 (Holmes, W. E. et al. Science 253:1278-80, 1991; Murphy, P. M. et al. Science 253:1280-3, 1991) have been cloned using molecular techniques. All of these receptors display amino acid sequences which are predicted to conform to an architecture containing seven-transmembrane-spanning segments connected by a series of intra- and extracellular loops. The primary sequences of these receptors revealed domains which were conserved in receptors associated with cell motility, but not in other seven-transmembrane-spanning receptors.

Accordingly, it is an object of the invention to provide isolated C-C chemokine receptor (C-C CKR-1) for use as a therapeutic or diagnostic reagent.

It is another object of the invention to make variants of C-C CKR-1 for use as antagonists or agonists.

It is another object of the invention to generate antibodies against C-C CKR-1 for use as diagnostic and therapeutic agents.

It is another object of the invention to provide a method for identifying new chemokine receptors.

SUMMARY OF THE INVENTION

One aspect of the invention is the isolation of the novel chemokine receptor, C-C CKR-1.

In another aspect, the invention provides a  
5 composition comprising C-C CKR-1 that is free of contaminating polypeptides of the animal species from which the C-C CKR-1 is derived.

In another aspect of the invention, C-C CKR-1, or fragments thereof (which also may be synthesized by  
10 chemical methods), is fused (by recombinant expression or in vitro covalent methods) to an immunogenic polypeptide and this fusion polypeptide, in turn, is used to immunize an animal to raise antibodies against a C-C CKR-1 epitope. Anti-C-C CKR-1 antibodies are recovered from the serum of  
15 immunized animals. Alternatively, monoclonal antibodies are prepared from cells of the immunized animal in conventional fashion.

Another aspect of the invention is the use of anti-C-C CKR-1 antibodies in the diagnosis of (in vitro or in vivo)  
20 or (when immobilized on an insoluble matrix) the purification of chemokine receptors which bind thereto.

Another aspect of the invention is the derivatization of C-C CKR-1 in vitro to prepare immobilized C-C CKR-1 and labeled C-C CKR-1, particularly for purposes of diagnosis  
25 of C-C CKR-1 or its antibodies, or for affinity purification of C-C CKR-1 antibodies themselves.

Another aspect of the invention is the formulation of C-C CKR-1, its derivatives, or its antibodies into physiologically acceptable vehicles, especially for  
30 therapeutic use. Such vehicles include sustained-release formulations of C-C CKR-1.

In still other aspects, the invention provides an isolated nucleic acid molecule encoding C-C CKR-1, labeled or unlabeled, and a nucleic acid sequence that is  
35 complementary to, or hybridizes under defined conditions to a nucleic acid sequence encoding C-C CKR-1.

In addition, the invention provides a replicable vector comprising the nucleic acid molecule encoding C-C CKR-1 operably linked to control sequences recognized by a

host transformed by the vector; host cells transformed with the vector; and a method of using a nucleic acid molecule encoding C-C CKR-1 to effect the production of C-C CKR-1, comprising expressing the nucleic acid molecule in a culture of the transformed host cells and recovering C-C CKR-1 from the host cell culture. The nucleic acid sequence is also useful in hybridization assays for C-C CKR-1 nucleic acid.

Another aspect of the invention is substitutional, deletional, or insertional variants of C-C CKR-1 amino acids and/or glycosyl residues, including variants having non-native glycosylation. These variants are prepared by in vitro or recombinant methods. Sequence variants are optionally screened for immuno-cross-reactivity with C-C CKR-1 and for C-C CKR-1 antagonist or agonist activity.

Another aspect of the invention is a method for identifying new C-C chemokine receptors.

Another aspect of the invention is a method for determining the biological activity of a C-C chemokine variant on C-C CKR-1, by transforming a host cell with DNA encoding C-C CKR-1, culturing the host cell to express the receptor on its surface, harvesting the cells, contacting the cells with a C-C chemokine variant, and determining the biological activity of the variant on the receptor.

In further embodiments, the invention provides transgenic animals comprising C-C CKR-1 from another species, animals in which C-C CKR-1 is expressed in a tissue in which it is not ordinarily found, or animals in which C-C CKR-1 is inactivated, by, for example, gene disruption.

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 (SEQ ID NOS:1-7) depicts the predicted amino acid sequence of C-C CKR-1, and alignment of the predicted amino acid sequence with HUMSTSR (Genbank accession #M99293), IL8rA (Holmes, W. E. et al. Science 253:1278-80, 1991), IL8rB (Murphy, P. M. et al. Science 253:1280-3, 1991), C5a receptor (Gerard, N. P. et al. Nature 349:614-7, 1991), fMLP receptor (Boulay, F. et al. Biochemistry

29:11123-11133, 1990) and the open reading frame of cytomegalovirus, US28. The seven putative transmembrane spanning domains are overlined. Glycosylation sites are indicated with black dots above the sequence. The two cysteine residues implicated in disulfide bonding are indicated with asterisks. The consensus site for protein kinase C phosphorylation is indicated as "+". Conserved amino acids appearing more than two times in the alignment are boxed.

10 Figure 2 depicts Northern blot analysis of RNA from hematopoietic cells probed with C-C CKR-1. 5 µg of poly A<sup>+</sup> or 20 µg of total RNA from the cell lines indicated was size fractionated on 1% formaldehyde-agarose, transferred to nitrocellulose and hybridized with radiolabeled C-C CKR-1 cDNA. The filter was washed with 0.5X SSC, 0.1% SDS at 15 55°C and the autoradiograph was developed after 4-6 hours exposure at -70°C with intensifying screens. RNA molecular weight markers were also run on the gel and are indicated on the side.

20 Figures 3A and 3B depict Southern blot analysis of human genomic DNA probed with C-C CKR-1. In Figure 3A, 10 µg of genomic DNA was digested with the restriction enzyme indicated, run on a 0.6% agarose gel, blotted onto Genescreen® and hybridized with radiolabeled C-C CKR-1 cDNA. The filter was washed with 0.5X SSC, 1% SDS at 25 55°C and the autoradiograph was developed after overnight exposure at -70°C with intensifying screens. DNA molecular weight markers were also run on the gel and are indicated on the side. In Figure 3B, the same blot was washed more 30 stringently with 0.2X SSC, 0.1% SDS at 60°C and autoradiography performed as indicated before.

Figures 4A and 4B are graphs depicting intracellular Ca<sup>++</sup> concentrations of 293 cells transfected with C-C CKR-1 cDNA and challenged with human MIP-1α (HuMIP-1α) and RANTES. 35 In Figure 4A, 50% confluent cells were transfected with 10-20 µg of plasmid DNA by the calcium-phosphate precipitation method. After transient expression for 12-24 hours, cells were harvested, loaded with the calcium probe INDO-1 AM and

assayed by spectrofluorometric methods at 37°C with continuous stirring. Various concentrations of HuMIP-1 $\alpha$ , as indicated, were added after 12 seconds. The intracellular concentrations of Ca<sup>++</sup> was determined as described (Naccache, P. H. et al. J. Immunol. 142:2438-44, 1989). In Figure 4B, details were as for Figure 4A, except that various concentration of RANTES, as indicated, were used.

Figures 5A-5D are graphs depicting desensitization in response to the challenge of the same or different ligands by 293 cells transiently expressing C-C CKR-1. Details are as described in Figure 4. The transfected cells were first challenged at 12 seconds with 100 nM of HuMIP-1 $\alpha$  or 250 nM of RANTES, and then at 70 seconds with the same concentration of ligands in the order indicated.

Figures 6A and 6B are graphs depicting the binding of <sup>125</sup>I-HuMIP-1 $\alpha$  and <sup>125</sup>I-RANTES on 293 cells transfected with C-C CKR-1 cDNA. In Figure 6A, Human embryonic kidney cells (293 cells) were transfected with 10-20  $\mu$ g plasmid DNA as described in Figure 4. Transfected cells were incubated for 2 hours at 4°C with <sup>125</sup>I-HuMIP-1 $\alpha$  in the presence of increasing concentrations of unlabeled HuMIP-1 $\alpha$ . The inset shows Scatchard analysis of the binding data and revealed a K<sub>d</sub> of 5.1 $\pm$ 0.3nM for <sup>125</sup>I-MIP-1 $\alpha$  to C-C CKR-1. Figure 6B depicts displacement of <sup>125</sup>I-RANTES with unlabeled HuMIP-1 $\alpha$  on 293 cells transfected with the C-C CKR-1 cDNA. Scatchard analysis of the binding data revealed a K<sub>d</sub> of 7.6 $\pm$ 1.5nM for the displacement of <sup>125</sup>I-RANTES to the C-C CKR-1.

Figure 7 is a graph depicting displacement of <sup>125</sup>I-HuMIP-1 $\alpha$  binding to 293 cells transfected with C-C CKR-1 cDNA. Cells were transfected as outlined in Figure 4 and incubated for 2 hours at 4°C with <sup>125</sup>I-HuMIP-1 $\alpha$  in the presence of increasing concentrations of the cross competing ligands, HuMIP-1 $\alpha$ , murine MIP-1 $\alpha$ , HuMIP-1 $\beta$ , MCP-1 and IL-8. The K<sub>d</sub> and the number of sites, shown in the bottom left corner, were determined by Scatchard analysis of the binding data.



Figure 8 is a graph depicting the intracellular  $\text{Ca}^{++}$  concentration of 293 cells transiently expressing C-C CKR-1 and challenged with HuMIP-1 $\alpha$ , RANTES, HuMIP-1 $\beta$  and MCP-1. Details are as described in Figure 4.

5        Figure 9 (SEQ ID NO:8) is the nucleotide sequence of C-C CKR-1 and its 3' noncoding region.

Figure 10 is a graph depicting the binding of radiolabeled HuMIP-1 $\alpha$  to 293 cells transfected with the coding region of the open reading frame US28 in the cytomegalovirus (CMV) genome. 293 cells were transfected with an expression construct containing the coding sequence of US28 in the sense or antisense orientation. After 12 hours, the cells were harvested and incubated with 0.9 nM of  $^{125}\text{I}$ -HuMIP-1 $\alpha$  in combination with 1  $\mu\text{M}$  of either  
10        unlabeled HuMIP-1 $\alpha$ , murine MIP-1 $\beta$ , MCP-1, RANTES, or IL-8. The amount of displaceable  $^{125}\text{I}$ -HuMIP-1 $\alpha$  was determined by subtracting the amount of  $^{125}\text{I}$ -HuMIP-1 $\alpha$  bound in the absence of any cold ligand from the amount bound in the presence of cold ligand. Background refers to counts  
15        obtained from cells transfected with the antisense orientation of US28.  
20       

#### DETAILED DESCRIPTION OF THE INVENTION

##### A. Definitions

25

In general, the following words or phrases have the indicated definition when used in the description, examples, and claims.

"C-C CKR-1" is the chemokine receptor described infra together with its amino acid sequence or cellular analogs, alleles, predetermined amino acid sequence mutations, glycosylation variants, and covalent modifications.

Embodiments of C-C CKR-1 exclude known chemokine receptors, in particular those which are set forth in the Background section above, and chemokine receptors statutorily obvious from those such chemokine receptors.  
30         
35       

"Orphan receptor" is defined as the predicted polypeptide encoded by nucleic acid which hybridizes under low stringency conditions to probes designed from known

cytokine receptor nucleic acid sequences or other known sequences likely to have structural similarity to cytokine receptors, or detectable by PCR primers so designed, wherein the predicted polypeptide is not previously known  
5 in the art.

"C-C CKR-1 qualitative biological activity" is defined as immunological cross-reactivity with at least one epitope of purified C-C CKR-1.

"Immunologically cross-reactive" is intended to mean  
10 that the candidate polypeptide is capable of competitively inhibiting the binding of native C-C CKR-1 to polyclonal antibodies or antisera raised against native C-C CKR-1, respectively.

"Isolated C-C CKR-1 nucleic acid or polypeptide" is a  
15 C-C CKR-1 nucleic acid or polypeptide that is identified and separated from at least one contaminant (nucleic acid or polypeptide respectively) with which it is ordinarily associated in nature, such as from the human source of C-C CKR-1 nucleic acid or polypeptide. In preferred  
20 embodiments, C-C CKR-1 will be isolated to pharmaceutically acceptable levels of purity with respect to proteins of its species of origin. In preferred embodiments, C-C CKR-1 protein will be purified (1) to greater than 95% by weight of protein, and most preferably more than 99% by weight,  
25 (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by an amino acid sequenator commercially available on the filing date hereof, or (3) to homogeneity by conventional nonreducing SDS polyacrylamide gel electrophoresis (SDS-PAGE) using  
30 Coomassie blue or, preferably, silver stain. Isolated C-C CKR-1 includes C-C CKR-1 in situ within recombinant cells which do not ordinarily express the C-C CKR-1 in question, since, in this instance, at least one component of C-C CKR-1 natural environment will not be present. Isolated C-C  
35 CKR-1 includes C-C CKR-1 in a recombinant cell culture of another species than the species of origin of the C-C CKR-1 since the C-C CKR-1 in such circumstances will be devoid of source polypeptides. Ordinarily, however, isolated C-C CKR-1 will be prepared by at least one purification step.

Isolated C-C CKR-1 nucleic acid includes a nucleic acid that is identified and separated from at least one containment nucleic acid with which it is ordinarily associated in the natural source of the C-C CKR-1 nucleic acid. Isolated C-C CKR-1 nucleic acid thus is present in other than in the form or setting in which it is found in nature. However, isolated C-C CKR-1-encoding nucleic acid includes C-C CKR-1 nucleic acid in ordinarily C-C CKR-1-expressing cells where the nucleic acid is in a chromosomal location different from that of natural cells or is otherwise flanked by a different DNA sequence than that found in nature.

The nucleic acid or polypeptide may be labeled for diagnostic and probe purposes, using a label as described and defined further below in the discussion of diagnostic assays.

"C-C CKR-1 nucleic acid" is defined as RNA or DNA (a) containing at least 25 bases of the genomic or cDNA sequence that encodes C-C CKR-1, (b) is complementary to the genomic or cDNA sequence that encodes C-C CKR-1, (c) which hybridizes to such nucleic acid and remains stably bound to it under stringent conditions, or (d) encodes a polypeptide sharing at least 50% sequence identity over the entire length of the polypeptide, preferably at least 60%, and more preferably at least 70%, with the amino acid sequence of C-C CKR-1, and which polypeptide has the ability to bind at least one C-C chemokine. Preferably the hybridizing RNA or DNA contains at least 25 bases, more preferably 40, and more preferably 60 bases which are identical to the sequences encoding the C-C CKR-1 described infra. Optimally, C-C CKR-1 nucleic acid consists essentially only of sequence encoding C-C CKR-1 or the complement of such sequences.

"Stringency" conditions for hybridization are defined by washing conditions after the hybridization reaction. Typically, hybridization conditions are defined as employing overnight incubation at 42°C, in a solution comprising 20% formamide, 5X SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5X

Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA. "High stringency" conditions for washing are defined as typically employing 0.2X SSC, 0.1% SDS at 55°C, while "low stringency"

5 conditions for washing are defined as typically employing 0.5X SSC, 1% SDS at 42°C. These conditions are well known in the art. See, for example, Current Protocols in Molecular Biology, eds. Ausubel, et al., Greene Publishing Associates, NY, 1989.

10 The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a  
15 ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory  
20 leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site  
25 is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However enhancers do not  
30 have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adapters or linkers are used in accord with conventional practice.

The starting plasmids used to practice this invention  
35 are commercially available, are publicly available on an unrestricted basis, or can be constructed from such available plasmids in accord with published procedures. In addition, other equivalent plasmids are known in the art and will be apparent to the ordinary artisan. Methods for

restriction enzyme digestion, recovery or isolation of DNA, hybridization analysis, and ligation are conventional and by this time well known to the ordinary artisan.

Similarly, the cell lines used to practice this invention  
5 are commercially available or are publicly available on an unrestricted basis.

Another method for obtaining the gene of interest is to chemically synthesize it using one of the methods described in Engels et al. (Agnew, Chem. Int. Ed. Engl.  
10 28:716-734, 1989). These methods include triester, phosphite, phosphoramidite and H-phosphonate methods, typically proceeding by oligonucleotide synthesis on solid supports.

"Recovery" or "isolation" of a given fragment of DNA  
15 from a restriction digest means separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the  
20 desired fragment, and separation of the gel from DNA. This procedure is known generally. For example, see Sambrook, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1989).

Amino acids are referred to by their standard three  
25 letter IUPAC abbreviations.

## B. General Methods for Practicing the Invention

### 1. Preparation of Native C-C CKR-1 Nucleic Acid

The use of the singular article "the" with respect to  
30 C-C CKR-1 is not intended to suggest that only one DNA sequence encodes C-C CKR-1. In fact, it is expected that alleles, processing intermediates, and predetermined sequence variants described infra will vary in sequence from the DNA encoding native C-C CKR-1. Further, C-C CKR-1  
35 may fall within a subfamily of chemokine receptors having a high degree of sequence homology but which vary sufficiently as to not constitute alleles. All of these sequences fall within the ambit of C-C CKR-1 nucleic acid.

DNA sequences encoding C-C CKR-1 may be either genomic

or cDNA. Any representative genomic library may be screened with the probes described below. Methods for genomic DNA preparation and the construction of cDNA libraries are well known in the art. See, for example, 5 Sambrook et al., supra.

## 2. Amino Acid Sequence Variants of C-C CKR-1

Amino acid sequence variants of C-C CKR-1 are prepared by introducing appropriate nucleotide changes into C-C CKR-1 DNA, or by in vitro synthesis of the desired C-C CKR-1 polypeptide. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence of native C-C CKR-1. Any combination of deletion, insertion, and substitution can be 10 made to arrive at the final construct, provided that the final construct possesses the desired characteristics. 15

The amino acid changes also may alter post-translational processing of C-C CKR-1, such as changing the number or position of glycosylation sites or by altering 20 its membrane anchoring characteristics.

In designing amino acid sequence variants of C-C CKR-1, the location of the mutation site and the nature of the mutation will depend on C-C CKR-1 characteristic(s) to be modified. The sites for mutation can be modified 25 individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting residues of the same or a different class adjacent to the located site, 30 or combinations of options 1-3.

A useful method for identification of certain residues or regions of C-C CKR-1 polypeptide that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (Science 35 244:1081-1085, 1989). Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids

with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to optimize the performance of a mutation at a given site, alanine scanning or random mutagenesis may be conducted at the target codon or region and the expressed C-C CKR-1 variants are screened for the optimal combination of desired activity.

C-C CKR-1 variants will exhibit at least a biological activity of the parental sequence, for example, chemokine binding or antigenic activity. Preferably, the antigenically active C-C CKR-1 is a polypeptide that binds to an antibody raised against the polypeptide in its native conformation, "native conformation" generally meaning the polypeptide as found in nature which has not been denatured by chaotropic agents, heat or other treatment that substantially modifies the three dimensional structure of the polypeptide (this can be determined, for example, by migration on nonreducing, nondenaturing sizing gels). Antibody used in determination of antigenic activity is rabbit polyclonal antibody raised by formulating the native non-rabbit polypeptide in Freund's complete adjuvant, subcutaneously injecting the formulation, and boosting the immune response by intraperitoneal injection of the formulation until the titer of anti-polypeptide antibody plateaus.

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably about 1 to 10 residues, and typically are contiguous. Preferably, deletions are made in regions of the protein that are the least conserved when C-C CKR-1 amino acid sequence is compared with other chemokine receptors. Such deletions will be more likely to modify the biological activity of the polypeptides more significantly than deletions made elsewhere. The number of consecutive deletions will be

selected so as to preserve the tertiary structure of C-C CKR-1 in the affected domain, e.g., beta pleated sheet or alpha helix.

Amino acid sequence insertions include amino- and/or  
5 carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e., insertions within C-C CKR-1 sequence) may range  
10 generally from about 1 to 10 residues, more preferably 1 to 5, most preferably 1 to 3.

Insertional variants of C-C CKR-1 or its extracellular segments include the fusion to the N- or C-terminus of C-C CKR-1 of immunogenic polypeptides, e.g., bacterial  
15 polypeptides such as  $\beta$ -lactamase or an enzyme encoded by the E. coli trp locus, or yeast protein, and C-terminal fusions with proteins having a long half-life such as in place of V<sub>H</sub> or V<sub>C</sub> domains of immunoglobulins comprising constant regions, albumin, or ferritin (for example, as  
20 described in WO 89/02922, published 6 April 1989).

Another group of variants are amino acid substitution variants. These variants have at least one amino acid residue in C-C CKR-1 molecule removed and a different residue inserted in its place. The sites of greatest  
25 interest for substitutional mutagenesis include sites identified as the active site(s) of C-C CKR-1, and sites where the amino acids found in C-C CKR-1 from various species are substantially different in terms of side-chain bulk, charge, and/or hydrophobicity.

30 Other sites of interest are those in which particular residues of C-C CKR-1 are conserved when compared with other chemokine receptors. These positions may be important for the biological activity of C-C CKR-1. These sites, especially those falling within a sequence of at  
35 least three other identically conserved sites, are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table I under the heading of preferred substitutions. If such substitutions result in a change in biological activity, then more



substantial changes, denominated exemplary substitutions in Table I, or as further described below in reference to amino acid classes, are introduced and the products screened.

5

Table I

	<u>Original Residue</u>	<u>Exemplary Substitutions</u>	<u>Preferred Substitutions</u>
10	Ala (A)	ser	ser
	Arg (R)	lys; gln; asn; ala	lys
	Asn (N)	gln; his; lys; arg; ala; asp	asp
15	Asp (D)	glu; asn; ala	asn
	Cys (C)	ser; ala; val	ala
	Gln (Q)	asn; glu; ala	asn; glu
	Glu (E)	asp; gln; ala	gln
	Gly (G)	ala; asn	ala
20	His (H)	asn; gln; lys; arg; ala	asn
	Ile (I)	leu; val; met; ala; phe	val
	Leu (L)	ile; val; met; ala; phe	met
	Lys (K)	arg; gln; asn; met; ala	arg
25	Met (M)	leu; phe; ile; ala	leu
	Phe (F)	leu; val; ile; ala; tyr	leu
	Pro (P)	ala	ala
	Ser (S)	thr; ala	ala
	Thr (T)	ser; val; ala	ser
30	Trp (W)	tyr; phe; ala	tyr
	Tyr (Y)	trp; phe; thr; ala; gln	phe
	Val (V)	ile; leu; met; phe; ala; thr	ala

35

Substantial modifications in function or immunological identity of C-C CKR-1 are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or

40 helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the

side chain. Naturally occurring residues are divided into groups based on common side chain properties:

- (1) hydrophobic: norleucine, met, ala, val, leu, ile;
- (2) neutral hydrophilic: cys, ser, thr;
- 5 (3) acidic: asp, glu;
- (4) basic: asn, gln, his, lys, arg;
- (5) residues that influence chain orientation: gly, pro;
- and
- (6) aromatic: trp, tyr, phe.

10 Non-conservative substitutions will entail exchanging a member of one of these classes for another. Such substituted residues may be introduced into regions of C-C CKR-1 that are homologous with other chemokine receptors, or, more preferably, into the non-homologous regions of the  
15 molecule.

Any cysteine residues not involved in maintaining the proper conformation of C-C CKR-1 may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant cross-linking.

20 DNA encoding amino acid sequence variants of C-C CKR-1 is prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by  
25 oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of C-C CKR-1. These techniques may utilize C-C CKR-1 nucleic acid (DNA or RNA), or nucleic acid complementary to C-C CKR-1 nucleic  
30 acid. Oligonucleotide-mediated mutagenesis is a preferred method for preparing substitution, deletion, and insertion variants of C-C CKR-1 DNA. This technique is well known in the art (see, for example, as described by Adelman et al., DNA 2:183, 1983). PCR mutagenesis is also suitable for  
35 making amino acid variants of C-C CKR-1 (see Erlich, supra, pp. 61-70). Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells et al. (Gene 34:315-323, 1985).

### 3. Insertion of DNA into a Cloning Vehicle

The cDNA or genomic DNA encoding native or variant C-C CKR-1 is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many  
5 vectors are available, and selection of the appropriate vector will depend on (1) whether it is to be used for DNA amplification or for DNA expression, (2) the size of the DNA to be inserted into the vector, and (3) the host cell to be transformed with the vector. Each vector contains  
10 various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more  
15 marker genes, an enhancer element, a promoter, and a transcription termination sequence.

#### a. Signal Sequence Component

In general, a signal sequence may be a component of  
20 the vector, or it may be a part of C-C CKR-1 DNA that is inserted into the vector.

#### b. Origin of Replication Component

Both expression and cloning vectors contain a nucleic  
25 acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating  
30 sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 $\mu$  plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or  
35 BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e. they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of C-C CKR-1 DNA. However, the recovery of genomic DNA encoding C-C CKR-1 is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise C-C CKR-1 DNA.

#### c. Selection Gene Component

Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g. ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g. the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern et al., J. Molec. Appl. Genet. 1:327-341, 1982), mycophenolic acid (Mulligan et al.,

Science 209:1422-1427, 1980) or hygromycin (Sugden et al.,  
Mol. Cell. Biol. 5:410-413, 1985). The three examples  
given above employ bacterial genes under eukaryotic control  
to convey resistance to the appropriate drug G418 or  
5 neomycin (geneticin), xgpt (mycophenolic acid), or  
hygromycin, respectively.

Another example of suitable selectable markers for  
mammalian cells are those that enable the identification of  
cells competent to take up C-C CKR-1 nucleic acid, such as  
10 dihydrofolate reductase (DHFR) or thymidine kinase. The  
mammalian cell transformants are placed under selection  
pressure which only the transformants are uniquely adapted  
to survive by virtue of having taken up the marker.  
Selection pressure is imposed by culturing the  
15 transformants under conditions in which the concentration  
of selection agent in the medium is successively changed,  
thereby leading to amplification of both the selection gene  
and the DNA that encodes C-C CKR-1. Amplification is the  
process by which genes in greater demand for the production  
20 of a protein critical for growth are reiterated in tandem  
within the chromosomes of successive generations of  
recombinant cells. Increased quantities of C-C CKR-1 are  
synthesized from the amplified DNA.

For example, cells transformed with the DHFR selection  
25 gene are first identified by culturing all of the  
transformants in a culture medium that contains  
methotrexate (Mtx), a competitive antagonist of DHFR. An  
appropriate host cell when wild-type DHFR is employed is  
the Chinese hamster ovary (CHO) cell line deficient in DHFR  
30 activity, prepared and propagated as described by Urlaub  
and Chasin, Proc. Natl. Acad. Sci. U.S.A., 77(7):4216-4220,  
1980). The transformed cells are then exposed to increased  
levels of methotrexate. This leads to the synthesis of  
multiple copies of the DHFR gene, and, concomitantly,  
35 multiple copies of other DNA comprising the expression  
vectors, such as the DNA encoding C-C CKR-1. This  
amplification technique can be used with any otherwise  
suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding  
the presence of endogenous DHFR if, for example, a mutant

DHFR gene that is highly resistant to Mtx is employed (EP 117,060). Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding C-C CKR-1, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418.

10 A suitable selection gene for use in yeast is the trp1 gene present in the yeast plasmid YRp7 (Stinchcomb et al., Nature 282:39-43, 1979); Kingsman et al., Gene 7:141-152, 1979); or Tschemper et al., Gene 10:157-166, 1980). The trp1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076. The presence of the trp1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, Leu2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the Leu2 gene.

#### e. Promoter Component

Expression vectors usually contain a promoter that is recognized by the host organism and is operably linked to C-C CKR-1 nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, such as C-C CKR-1, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g. the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to DNA encoding

C-C CKR-1 by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native C-C CKR-1 promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of C-C CKR-1 DNA. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of expressed C-C CKR-1 as compared to the native C-C CKR-1 promoter.

Promoters suitable for use with prokaryotic hosts include the  $\beta$ -lactamase and lactose promoter systems (Chang et al., Nature 275:617-624, 1978); and Goeddel et al., Nature 281:544-548, 1979), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, Nucleic Acids Res. 8(18):4057-4074, 1980) and EP 36,776) and hybrid promoters such as the tac promoter (deBoer et al., Proc. Natl. Acad. Sci. U.S.A. 80:21-25, 1983). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker to operably ligate them to DNA encoding C-C CKR-1 (Siebenlist et al., Cell 20:269-281, 1980) using linkers or adapters to supply any required restriction sites. Promoters for use in bacterial systems also generally will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding C-C CKR-1.

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem. 255(24):12073-80, 1980) or other glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg. 7:149-67, 1968); and Holland, Biochemistry 17:4900-4907, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase,

degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman et al., EP 73,657A. Yeast enhancers also are advantageously used with yeast promoters.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into mammalian expression vectors.

C-C CKR-1 transcription from vectors in mammalian host cells is controlled by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g. the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with C-C CKR-1 sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication (Fiers et al., Nature 273:113-120, 1978; Mulligan and Berg, Science 209:1422-1427, 1980; Pavlakis et al., Proc. Natl. Acad. Sci. U.S.A. 78:7398-7402, 1981). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment (Greenaway et al., Gene 18:355-360, 1982). A system for expressing DNA in



mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. 4,419,446. A modification of this system is described in U.S. 4,601,978. See also Gray et al., Nature 295:503-508, 1982, on expressing cDNA encoding immune interferon in monkey cells; Reyes et al., Nature 297:598-601, 1982, on expression of human  $\beta$ -interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus, Canaani and Berg, Proc. Natl. Acad. Sci. U.S.A. 79:5166-5170, 1982, on expression of the human interferon  $\beta$ 1 gene in cultured mouse and rabbit cells, and Gorman et al., Proc. Natl. Acad. Sci. U.S.A. 79:6777-6781, 1982, on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

f. Enhancer Element Component

Transcription of a DNA encoding C-C CKR-1 of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10-300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent having been found 5' (Laimins et al., Proc. Natl. Acad. Sci. U.S.A. 78:464-8, 1981) and 3' (Lusky et al., Mol. Cell Bio. 3(6):1108-1122, 1983) to the transcription unit, within an intron (Banerji et al., Cell 33:729-740, 1983) as well as within the coding sequence itself (Osborne et al., Mol. Cell Bio. 4(7):1293-1305, 1984). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin,  $\alpha$ -fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature 297:17-18, 1982, on enhancing elements for activation of

eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to C-C CKR-1 DNA, but is preferably located at a site 5' from the promoter.

5                   g. Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription  
10 and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the  
15 mRNA encoding C-C CKR-1. The 3' untranslated regions also include transcription termination sites.

Suitable vectors containing one or more of the above listed components and the desired coding and control sequences are constructed by standard ligation techniques.  
20 Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform E.  
25 coli K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of  
30 Messing et al., Nucleic Acids Res. 9(2):309-321, 1981, or by the method of Maxam et al., Methods in Enzymology 65:499-560, 1980.

Particularly useful in the practice of this invention are expression vectors that provide for the transient  
35 expression in mammalian cells of DNA encoding C-C CKR-1. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes

high levels of a desired polypeptide encoded by the expression vector. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of C-C CKR-1 that have C-C CKR-1-like activity, and for analysis of the effect of the binding of chemokine variants to C-C CKR-1.

Other methods, vectors, and host cells suitable for adaptation to the synthesis of C-C CKR-1 in recombinant vertebrate cell culture are described in Gething et al., Nature 293:620-625, 1981; Mantei et al., Nature 281:40-46, 1979); EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of C-C CKR-1 is pRK5 (EP pub. no. 307,247) or pSVI6B (U.S. Ser. No. 07/441,574 filed 22 November 1989).

20

#### 4. Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing C-C CKR-1 expression vectors are the prokaryote, yeast, or higher eukaryotic cells described above. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *E. coli*, *Bacilli* such as *B. subtilis*, *Pseudomonas* species such as *P. aeruginosa*, *Salmonella typhimurium*, or *Serratia marcescens*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli*  $\chi$ 1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting. Preferably the host cell should secrete minimal amounts of proteolytic enzymes. Alternatively, in vitro methods of cloning, e.g. PCR or other nucleic acid polymerase reactions, are suitable.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for vectors containing C-C CKR-1 DNA. *Saccharomyces*

cerevisiae, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful to practice the invention, such as S. pombe (Beach and Nurse, Nature 290:140-143, 1981), Kluyveromyces lactis (Louvencourt et al., J. Bacteriol. 154(2):737-742, 1983), Pichia pastoris (EP 183,070), Trichoderma reesia (EP 244,234), Neurospora crassa (Case et al., Proc. Natl. Acad. Sci. U.S.A. 76:5259-5263, 1979), and Aspergillus hosts such as A. nidulans (Ballance et al., Biochem. Biophys. Res. Commun. 112:284-289, 1983); Tilburn et al., Gene 26:205-221, 1983); Yelton et al., Proc. Natl. Acad. Sci. U.S.A. 81:1470-1474, 1984) and A. niger (Kelly and Hynes, EMBO J. 4:475-479, 1985).

Suitable host cells for the expression of glycosylated C-C CKR-1 polypeptide are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as Spodoptera frugiperda (caterpillar); Aedes aegypti (mosquito), Aedes albopictus (mosquito), Drosophila melanogaster (fruit fly), and Bombyx mori host cells have been identified. See, e.g., Luckow et al., Bio/Technology 6:47-55, 1988); Miller et al., in Genetic Engineering, Setlow, J.K. et al., 8:277-279 (Plenum Publishing, 1986), and Maeda et al., Nature 315:592-594, 1985). A variety of such viral strains are publicly available, e.g., the L-1 variant of Autographa californica NPV and the Bm-5 strain of Bombyx mori NPV, and such viruses may be used as the virus according to the present invention, particularly for transfection of Spodoptera frugiperda cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium Agrobacterium tumefaciens,

which has been previously manipulated to contain C-C CKR-1 DNA. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding C-C CKR-1 is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express C-C CKR-1 DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker et al., J. Mol. Appl. Gen. 1: 561-573, 1982). In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. See EP 321,196 published 21 June 1989.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (Tissue Culture, Academic Press, Kruse and Patterson, eds., 1973). Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen. Virol. 36:59-72, 1977); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. U.S.A. 77(7):4216-4220, 1980); mouse sertoli cells (TM4, Mather, Biol. Reprod. 23:243-251, 1980); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HeLa, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci. 383:44-68, 1982); MRC 5 cells; FS4 cells; and a human hepatoma cell line (Hep G2). Preferred host cells are human embryonic kidney 293 and Chinese hamster ovary cells.

The host chosen for expression may also be a multicellular organism, as in a transgenic animal. Such

animals have been produced by transfection of germ cells, somatic cells, or embryos with heterologous DNA, suitably implanting the transfected cells and allowing the cells to mature into or stably integrate into adult animals

5 containing the heterologous DNA. A reproducible percentage of such animals transcribe and express the heterologous DNA as protein which can be identified in tissues including blood or serum. Suitable methods for making transgenic animals are described in U.S. Patent 4,396,601 and Palmiter  
10 et al., Nature 300:611-615, 1982.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting  
15 transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection  
20 are known to the ordinarily skilled artisan, for example,  $\text{CaPO}_4$  and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism  
25 so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described  
30 in section 1.82 of Sambrook et al., is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., Gene 23:315-330, 1983) and WO  
35 89/05859, published 29 June 1989. For mammalian cells without such cell walls, the calcium phosphate precipitation method described in sections 16.30-16.37 of Sambrook et al., supra, is preferred. General aspects of mammalian cell host system transformations have been

described by Axel in U.S. 4,399,216, issued 16 August 1983. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bacteriol. 130(2):946-947, 1977) and Hsiao et al., Proc. Natl. Acad. Sci. U.S.A., 76(8):3829-3833, 1979). However, other methods for introducing DNA into cells such as by nuclear injection, electroporation, or by protoplast fusion may also be used.

10           5. Culturing the Host Cells

Prokaryotic cells used to produce C-C CKR-1 polypeptide of this invention are cultured in suitable media as described generally in Sambrook et al., supra.

15           The mammalian host cells used to produce C-C CKR-1 of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium (MEM, Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium (DMEM, Sigma) are  
20           suitable for culturing the host cells. In addition, any of the media described in Ham and McKeehan, Meth. Enz. 58:44-93, 1979, Barnes and Sato, Anal. Biochem. 102:255-270, 1980, U.S. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Pat. Re. 30,985; or U.S.  
25           5,122,469 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate),  
30           buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin<sup>TM</sup> drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary  
35           supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the

ordinarily skilled artisan.

The host cells referred to in this disclosure encompass cells in in vitro culture as well as cells that are within a host animal.

5

#### 6. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, northern blotting to quantitate the transcription of mRNA (Thomas, Proc. Natl. Acad. Sci. U.S.A. 10 77:5201-5205, 1980), dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe. Various labels may be employed, most commonly radioisotopes, particularly  $^{32}\text{P}$ . However, other techniques 15 may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the 20 like.

Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the 25 assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by 30 immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and 35 fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use



in the present invention is described by Hsu et al.,  
Am. J. Clin. Path. 75:734-738, 1980.

Antibodies useful for immunohistochemical staining  
and/or assay of sample fluids may be either monoclonal or  
5 polyclonal, and may be prepared in any mammal.  
Conveniently, the antibodies may be prepared against a  
native or synthetic C-C CKR-1 polypeptide or variant  
thereof.

10        7. Purification of C-C CKR-1 Polypeptide

C-C CKR-1 is recovered from cell cultures by  
solubilizing cell membranes in detergent.

When a human C-C CKR-1 is expressed in a recombinant  
cell other than one of human origin, C-C CKR-1 is  
15 completely free of proteins or polypeptides of human  
origin. However, it is necessary to purify C-C CKR-1 from  
recombinant cell proteins or polypeptides to obtain  
preparations that are substantially homogeneous by protein  
as to C-C CKR-1. As a first step, the cells are  
20 centrifuged to separate them from culture medium, followed  
by suitable purification procedures such as: fractionation  
on immunoaffinity or ion-exchange columns; ethanol  
precipitation; reverse phase HPLC; chromatography on silica  
or on a cation exchange resin such as DEAE;  
25 chromatofocusing; SDS-PAGE; ammonium sulfate precipitation;  
gel filtration using, for example, Sephadex G-75.

C-C CKR-1 variants in which residues have been  
deleted, inserted or substituted are recovered in the same  
fashion as the native C-C CKR-1, taking account of any  
30 substantial changes in properties occasioned by the  
variation. For example, preparation of a C-C CKR-1 fusion  
with another protein or polypeptide, e.g. a bacterial or  
viral antigen, facilitates purification; an immunoaffinity  
column containing antibody to the antigen can be used to  
35 adsorb the fusion. Immunoaffinity columns such as a rabbit  
polyclonal anti-C-C CKR-1 column can be employed to absorb  
C-C CKR-1 variant by binding it to at least one remaining  
immune epitope. A protease inhibitor such as phenyl methyl  
sulfonyl fluoride (PMSF) also may be useful to inhibit

proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native C-  
5 C CKR-1 may require modification to account for changes in the character of C-C CKR-1 or its variants upon expression in recombinant cell culture.

#### 8. Covalent Modifications of C-C CKR-1 Polypeptides

10 Covalent modifications of C-C CKR-1 polypeptide or its glycosyl substituents are included within the scope of this invention. Both native C-C CKR-1 and amino acid sequence variants of C-C CKR-1 may be covalently modified. Covalent  
15 modifications of C-C CKR-1, fragments thereof or antibodies thereto are introduced into the molecule by reacting targeted amino acid residues of C-C CKR-1, fragments thereof, or C-C CKR-1 antibody with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues. Most commonly, C-C CKR-1  
20 and its antibodies are covalently bonded to detectable groups used in diagnosis, e.g. enzymes, radio isotopes, spin labels, antigens, fluorescent or chemiluminescent groups and the like.

Cysteiny l residues most commonly are reacted with  $\alpha$ -  
25 haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteiny l residues also are derivatized by reaction with bromotrifluoroacetone,  $\alpha$ -bromo- $\beta$ -(5-imidazole)propionic acid, chloroacetyl  
30 phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with  
35 diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysiny l and amino terminal residues are reacted with

succinic or other carboxylic acid anhydrides.

Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing  $\alpha$ -amino-containing

5 residues include imidoesters such as methyl picolinimate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

10 Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the  
15 high  $pK_a$  of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral  
20 labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using  $^{125}\text{I}$  or  
25  $^{131}\text{I}$  to prepare labeled proteins for use in radioimmunoassay, the chloramine T method described above being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides ( $\text{R}'\text{-N}=\text{C}=\text{N-R}$ ), where R and R' are different alkyl groups, such  
30 as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with  
35 ammonium ions.

Derivatization with bifunctional agents is useful for cross-linking C-C CKR-1, its fragments or antibodies to a water-insoluble support matrix or surface for use in methods for purifying anti-C-C CKR-1 antibodies, and vice

versa. Commonly used cross-linking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis-(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimide yield photoactivatable intermediates that are capable of forming cross-links in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Glutaminy and asparaginy residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the  $\alpha$ -amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 1983), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of C-C CKR-1 polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. By altering is meant deleting one or more carbohydrate moieties found in the native polypeptide, and/or adding one or more glycosylation sites that are not present in the native polypeptide.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tri-peptide sequences asparagine-X-serine and

asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tri-peptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose, to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to C-C CKR-1 polypeptide is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tri-peptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native C-C CKR-1 sequence (for O-linked glycosylation sites). For ease, C-C CKR-1 amino acid sequence is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding C-C CKR-1 polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above under the heading of "Amino Acid Sequence Variants of C-C CKR-1 Polypeptide".

Another means of increasing the number of carbohydrate moieties on C-C CKR-1 polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. These procedures are advantageous in that they do not require production of the polypeptide in a host cell that has glycosylation capabilities for N- and O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September

1987, and in Aplin and Wriston (CRC Crit. Rev. Biochem. pp. 259-306, 1981).

Removal of carbohydrate moieties present on the native C-C CKR-1 polypeptide may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin et al. (Arch. Biochem. Biophys. 259:52-57, 1987) and by Edge et al. (Anal. Biochem. 118:131-137, 1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo- glycosidases as described by Thotakura et al. (Meth. Enzymol. 138:350-359, 1987).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin et al. (J. Biol. Chem. 257:3105-3109, 1982). Tunicamycin blocks the formation of protein-N-glycoside linkages.

The C-C CKR-1 also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-[methylmethacrylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th edition, Osol, A., ed., 1980).

C-C CKR-1 preparations are also useful in generating antibodies, for use as standards in assays for C-C CKR-1 (e.g. by labeling C-C CKR-1 for use as a standard in a radioimmunoassay, enzyme-linked immunoassay, or radioreceptor assay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with radioiodine, enzymes, fluorophores, spin labels, and the like.

Since it is often difficult to predict in advance the characteristics of a variant C-C CKR-1, it will be appreciated that some screening of the recovered variant will be needed to select the optimal variant. For example, a change in the immunological character of C-C CKR-1 molecule, such as affinity for a given antibody, is measured by a competitive-type immunoassay. The variant is assayed for changes in the suppression or enhancement of its activity by comparison to the activity observed for native C-C CKR-1 in the same assay. Other potential modifications of protein or polypeptide properties such as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, or the tendency to aggregate with carriers or into multimers are assayed by methods well known in the art.

#### 9. Therapeutic Compositions and Administration of C-C CKR-1

Therapeutic formulations of C-C CKR-1 (including its C-C CKR-1 binding fragments) or antibodies thereto are prepared for storage by mixing C-C CKR-1 having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences, supra), in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronic, or polyethylene glycol (PEG).

The C-C CKR-1 or antibody to be used for in vivo

administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. The C-C CKR-1 ordinarily will be stored in  
5 lyophilized form or in solution.

Therapeutic C-C CKR-1 or antibody compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection  
10 needle.

The route of C-C CKR-1 or antibody administration is in accord with known methods, e.g. injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, or intralesional routes, or by  
15 sustained release systems as noted below.

Suitable examples of sustained-release preparations include semipermeable polymer matrices in the form of shaped articles, e.g. films, or microcapsules. Sustained release matrices include polyesters, hydrogels,  
20 polylactides (U.S. 3,773,919, EP 58,481), copolymers of L-glutamic acid and  $\gamma$  ethyl-L-glutamate (Sidman et al., Biopolymers 22:547-556, 1983), poly (2-hydroxyethyl-methacrylate) (Langer et al., J. Biomed. Mater. Res. 15:167-277, 1981; Langer, Chem. Tech., 12:98-105, 1982),  
25 ethylene vinyl acetate (Langer et al., supra) or poly-D-(-)-3-hydroxybutyric acid (EP 133,988). Sustained-release C-C CKR-1 or antibody compositions also include liposomally entrapped C-C CKR-1 or antibody. Liposomes containing C-C CKR-1 or antibody are prepared by methods known per se: DE  
30 3,218,121; Epstein et al., Proc. Natl. Acad. Sci. U.S.A. 82:3688-3692, 1985; Hwang et al., Proc. Natl. Acad. Sci. U.S.A. 77:4030-4034, 1980; EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. 4,485,045 and 4,544,545; and EP 102,324.  
35 Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal C-C CKR-1 or antibody therapy.



An effective amount of C-C CKR-1 or antibody to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. For example, it is expected that C-C CKR-1 will be therapeutically effective in the treatment of cytokine-mediated inflammation. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. Typically, the clinician will administer C-C CKR-1 or antibody until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored by conventional assays.

#### 10. C-C CKR-1 Antibody Preparation

Polyclonal antibodies to C-C CKR-1 generally are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of C-C CKR-1 and an adjuvant. Immunization with recombinant cells transformed with C-C CKR-1 (e.g. mouse or CHO cells transformed with human C-C CKR-1) may be satisfactory, or it may be useful to separate C-C CKR-1 and conjugate it or a fragment containing the target amino acid sequence to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride,  $\text{SOCl}_2$ , or  $\text{R}^1\text{N} = \text{C} = \text{NR}$ , where R and  $\text{R}^1$  are different alkyl groups.

Animals ordinarily are immunized against the cells or immunogenic conjugates or derivatives by combining 1 mg or 1  $\mu\text{g}$  of C-C CKR-1 in Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. 7 to

14 days later animals are bled and the serum is assayed for anti-C-C CKR-1 titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same C-C CKR-1, but conjugated to a different protein and/or through a different cross-linking agent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

Another option is to employ combinatorial variable domain libraries and screening methods to identify the desired anti-C-C CKR-1 antibodies.

Monoclonal antibodies are prepared by recovering spleen cells from immunized animals and immortalizing the cells in conventional fashion, e.g. by fusion with myeloma cells or by Epstein-Barr (EB)-virus transformation and screening for clones expressing the desired antibody.

The monoclonal antibody preferably is specific for each target C-C CKR-1 polypeptide. The antibody is selected to be either agonistic, antagonistic or to have no effect on the activity of or binding of the C-C CKR-1.

#### 11. Uses of C-C CKR-1 Nucleic Acid, and Antibodies

The nucleic acid encoding C-C CKR-1 may be used as a diagnostic for tissue specific typing. For example, such procedures as in situ hybridization, and northern and Southern blotting, and PCR analysis may be used to determine whether DNA and/or RNA encoding C-C CKR-1 are present in the cell type(s) being evaluated.

Isolated C-C CKR-1 polypeptide may be used in quantitative diagnostic assays as a standard or control against which samples e.g., from erythrocytes, containing unknown quantities of C-C CKR-1 may be compared. Recombinant cells which express C-C CKR-1 can be used in assays for C-C CKR-1 ligands in the same fashion as for example neutrophils are used in IL-8 assays. The C-C CKR-1 polypeptides, fragments or cells (as such, or derivatized) also can be used as immunogens in the production of antibodies to C-C CKR-1, or for the purification of such antibodies from ascites or recombinant cell culture media.

C-C CKR-1 antibodies are useful in diagnostic assays for C-C CKR-1 expression in specific cells or tissues wherein the antibodies are labeled in the same fashion as C-C CKR-1 described above and/or are immobilized on an insoluble matrix. C-C CKR-1 antibodies also are useful for the affinity purification of C-C CKR-1 from recombinant cell culture or natural sources. The C-C CKR-1 antibodies that do not detectably cross-react with other chemokine receptors can be used to purify each C-C CKR-1 free from other homologous chemokine receptors. C-C CKR-1 antibodies that are PF4 superfamily antagonists are useful as anti-inflammatory agents or in the treatment of other PF4 superfamily-mediated disorders.

Suitable diagnostic assays for C-C CKR-1 and its antibodies are well known per se. Such assays include competitive and sandwich assays, and steric inhibition assays. Competitive and sandwich methods employ a phase-separation step as an integral part of the method while steric inhibition assays are conducted in a single reaction mixture. Fundamentally, the same procedures are used for the assay of C-C CKR-1 and for substances that bind C-C CKR-1, although certain methods will be favored depending upon the molecular weight of the substance being assayed. Therefore, the substance to be tested is referred to as an analyte, irrespective of its status otherwise as an antigen or antibody, and proteins that bind to the analyte are denominated binding partners, whether they be antibodies, cell surface receptors, or antigens.

Analytical methods for C-C CKR-1 or its antibodies all use one or more of the following reagents: labeled analyte analog, immobilized analyte analog, labeled binding partner, immobilized binding partner and steric conjugates. The labeled reagents also are known as "tracers."

The label used (and this is also useful to label C-C CKR-1 nucleic acid for use as a probe) is any detectable functionality that does not interfere with the binding of analyte and its binding partner. Numerous labels are known for use in immunoassay, examples including moieties that may be detected directly, such as fluorochrome,

chemiluminescent, and radioactive labels, as well as moieties, such as enzymes, that must be reacted or derivatized to be detected. Examples of such labels include the radioisotopes  $^{32}\text{P}$ ,  $^{14}\text{C}$ ,  $^{125}\text{I}$ ,  $^3\text{H}$ , and  $^{131}\text{I}$ ,  
5 fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luciferases, e.g., firefly luciferase and bacterial luciferase (U.S. Pat. No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP),  
10 alkaline phosphatase,  $\beta$ -galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to  
15 oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free radicals, and the like.

Conventional methods are available to bind these labels covalently to proteins or polypeptides. For  
20 instance, coupling agents such as dialdehydes, carbodiimides, dimaleimides, bis-imidates, bis-diazotized benzidine, and the like may be used to tag the antibodies with the above-described fluorescent, chemiluminescent, and enzyme labels. See, for example, U.S. Pat. Nos. 3,940,475  
25 (fluorimetry) and 3,645,090 (enzymes); Hunter et al., Nature 194:495-496, 1962; David et al., Biochemistry 13:1014-1021, 1974; Pain et al., J. Immunol. Methods 40:219-230, 1981; and Nygren, J. Histochem. and Cytochem. 30:407-412, 1982. Preferred labels are enzymes such as  
30 horseradish peroxidase and alkaline phosphatase.

The conjugation of such label, including the enzymes, to the antibody is a standard manipulative procedure for one of ordinary skill in immunoassay techniques. See, for example, O'Sullivan et al., "Methods for the Preparation of  
35 Enzyme-antibody Conjugates for Use in Enzyme Immunoassay," in Methods in Enzymology, ed. J.J. Langone and H. Van Vunakis, Vol. 73 (Academic Press, New York, New York, 1981), pp. 147-166. Such bonding methods are suitable for use with C-C CKR-1 or its antibodies, all of which are

proteinaceous.

Immobilization of reagents is required for certain assay methods. Immobilization entails separating the binding partner from any analyte that remains free in solution. This conventionally is accomplished by either insolubilizing the binding partner or analyte analog before the assay procedure, as by adsorption to a water-insoluble matrix or surface (Bennich et al., U.S. 3,720,760), by covalent coupling (for example, using glutaraldehyde cross-linking), or by insolubilizing the partner or analog afterward, e.g., by immunoprecipitation.

Other assay methods, known as competitive or sandwich assays, are well established and widely used in the commercial diagnostics industry.

Competitive assays rely on the ability of a tracer analog to compete with the test sample analyte for a limited number of binding sites on a common binding partner. The binding partner generally is insolubilized before or after the competition and then the tracer and analyte bound to the binding partner are separated from the unbound tracer and analyte. This separation is accomplished by decanting (where the binding partner was preinsolubilized) or by centrifuging (where the binding partner was precipitated after the competitive reaction). The amount of test sample analyte is inversely proportional to the amount of bound tracer as measured by the amount of marker substance. Dose-response curves with known amounts of analyte are prepared and compared with the test results to quantitatively determine the amount of analyte present in the test sample. These assays are called ELISA systems when enzymes are used as the detectable markers.

Another species of competitive assay, called a "homogeneous" assay, does not require a phase separation. Here, a conjugate of an enzyme with the analyte is prepared and used such that when anti-analyte binds to the analyte the presence of the anti-analyte modifies the enzyme activity. In this case, C-C CKR-1 or its immunologically active fragments are conjugated with a bifunctional organic bridge to an enzyme such as peroxidase. Conjugates are

selected for use with anti-C-C CKR-1 so that binding of the anti-C-C CKR-1 inhibits or potentiates the enzyme activity of the label. This method per se is widely practiced under the name of EMIT.

- 5       Steric conjugates are used in steric hindrance methods for homogeneous assay. These conjugates are synthesized by covalently linking a low-molecular-weight hapten to a small analyte so that antibody to hapten substantially is unable to bind the conjugate at the same time as anti-analyte.
- 10       Under this assay procedure the analyte present in the test sample will bind anti-analyte, thereby allowing anti-hapten to bind the conjugate, resulting in a change in the character of the conjugate hapten, e.g., a change in fluorescence when the hapten is a fluorophore.
- 15       Sandwich assays particularly are useful for the determination of C-C CKR-1 or C-C CKR-1 antibodies. In sequential sandwich assays an immobilized binding partner is used to adsorb test sample analyte, the test sample is removed as by washing, the bound analyte is used to adsorb
- 20       labeled binding partner, and bound material is then separated from residual tracer. The amount of bound tracer is directly proportional to test sample analyte. In "simultaneous" sandwich assays the test sample is not separated before adding the labeled binding partner. A
- 25       sequential sandwich assay using an anti-C-C CKR-1 monoclonal antibody as one antibody and a polyclonal anti-C-C CKR-1 antibody as the other is useful in testing samples for C-C CKR-1 activity.

      The foregoing are merely exemplary diagnostic assays

30       for C-C CKR-1 and antibodies. Other methods now or hereafter developed for the determination of these analytes are included within the scope hereof, including the bioassays described above.

      The following examples are offered by way of

35       illustration and not by way of limitation.

### C. EXPERIMENTAL EXAMPLES

#### Example I

Isolation of Orphan Receptors

An "orphan receptor" cloning strategy was employed in an attempt to isolate cDNAs encoding C-C chemokine receptors. The cell surface receptors for the chemoattractants C5a (Gerard, N. P. et al. Nature 349:614-7, 1991), the bacterial tripeptide fMLP (Boulay, F. et al. Biochemistry 29:11123-11133, 1990), as well as two receptors for the C-X-C chemokine IL-8 receptors A and B (IL8rA and IL8rB) have been recently cloned (Holmes, W. E. et al. Science 253:1278-80, 1991; Murphy, P. M. et al. Science 253:1280-3, 1991) and found to belong to the superfamily of receptor proteins whose structures are predicted to transverse the cell membrane seven times (Dohlman, H. G. et al. Annu. Rev. Biochem. 60:653-88, 1991). Since seven-transmembrane-spanning molecules are typically linked to G-proteins whose function can be inhibited by pertussis toxin, we assumed that the receptors for the C-C chemokine class of proteins would also share the seven transmembrane architecture. Accordingly, two degenerate oligonucleotides corresponding to conserved amino acid sequences in two transmembrane regions (TM) of the IL8rA, the C5a and the fMLP receptors were synthesized. The first oligonucleotide corresponded to a region in TM2: LNLA(L/V)AD(L/F)(L/G) (SEQ ID NO:9) and the second in TM7: NP(I/M)(I/L)Y(A/V)(F/V)(I/M/A)GQ (SEQ ID NO:10).

These oligomers were then used as primers in RT-PCR experiments using cDNA substrates from different hematopoietic cell types known to respond to C-C chemokines including peripheral blood mononuclear cells (PBMC), and the cell lines U937, HL60 and THP-1. PCR was performed as follows. 1-2 µg of total RNA from different hematopoietic cell lines were used as substrates in RT-PCR (Larrick, J. W. Trends Biotech. 10:146-152, 1992), as recommended by the supplier (Perkin Elmer, Norwalk, CT). Degenerate oligonucleotides corresponding to conserved regions of chemoattractant receptors were used in the PCR. PCR conditions were as follows: 94°C for 0.5', 50-55°C for 0.5', 72°C for 0.5-1', 30 cycles. PCR products were blunt-end cloned into the SmaI site of pBS (Stratagene, LaJolla, CA)

as previously described (Nguyen, T. et al. Gene 109:211-8, 1991). Plasmid DNA was isolated using the Quiagen kit (Quiagen Inc., Chatsworth, CA) as recommended by the supplier. Sequencing was performed with the Sequenase kit  
5 (USBC, Cleveland, OH) as recommended by the supplier.

Subcloning and sequencing of the PCR products revealed the presence of IL8rA, IL8rB, C5a receptor and two novel clones having characteristics of seven-transmembrane-segment receptors and marked similarity to the two IL-8  
10 receptors. Partial functional characterization of these orphan receptors revealed that they do not bind to HuMIP-1 $\alpha$ . However, it was noted that these two clones, which were more related to the IL-8 receptors than to other seven-transmembrane-spanning receptors, possessed a new  
15 conserved amino acid motif at the end of TM3, DRYLAIVHA (SEQ ID NO:11), which seemed to define a subfamily of IL-8 receptor-related seven-transmembrane-spanning molecules that excluded the C5a and the fMLP receptors. Therefore, a second round of RT-PCR/orphan cloning was carried out using  
20 the TM2 degenerate oligonucleotide and a DRYLAIVHA (SEQ ID NO:11) degenerate oligonucleotide. In addition, to increase the chances of obtaining C-C chemokine receptors, cDNA was obtained from cultured human monocytes, which bind radiolabeled HuMIP-1 $\alpha$  and from B Cells, which respond  
25 chemotactically to HuMIP-1 $\alpha$  and used in the PCR reaction. Cloning and sequencing of these PCR products revealed several additional unique seven-transmembrane-spanning receptors.

Monocytes were cultured by standard techniques  
30 commonly known in the art. In summary, buffy coat cells were separated on a Ficoll gradient. Mononuclear cells recovered from the interface were repeatedly centrifuged to remove platelets. The monocytes were separated from other mononuclear cells by adhering them to tissue culture  
35 dishes. The non-adherent cells were washed off and the adherent monocytes were then were cultured for 48-72 hours before use.

Alternatively, genomic or cDNA could be screened for



the presence of orphan receptor genes by traditional Southern blot hybridization to the oligomers described above, or to probes designed from cloned DNA of a seven-transmembrane spanning protein.

5

#### Example II

##### Characterization of C-C CKR-1 DNA

A cDNA corresponding to one clone, JOSH1, was isolated by screening a  $\lambda$ gt10 cDNA library made from PMA (phorbol 12-myristate 13-acetate) treated HL60 cells with  $^{32}$ P-labeled restriction fragments. Phage DNA was isolated and the inserts of the  $\lambda$ gt10 clones were obtained by PCR, employing 20 cycles with primers flanking the insert and the enzyme Pfu DNA polymerase (Stratagene, LaJolla, CA), as recommended by the supplier. The PCR products were subcloned into the SmaI site of pRK5 as described above.

The nucleotide sequence of JOSH1 revealed an open reading frame of 1065 bases, encoding a protein of 355 amino acids (Figures 1 and 9). The deduced amino acid sequence, provisionally designated as the C-C chemokine receptor 1 (C-C CKR-1), has key features related to G-protein-linked receptors of the seven-transmembrane-spanning receptor superfamily. For example, it has seven hydrophobic regions predicted to span the cell membrane, and cysteine residues in the first and the second extracellular loops that are implicated in forming a disulfide bond (Figure 1). However, certain features of the predicted C-C CKR-1 protein make it distinct from classical seven-transmembrane-spanning receptors. The carboxyl-terminus is relatively short and lacks cysteine residues involved in membrane anchorage via a palmitoylated moiety (O'Dowd, B. F. et al. J. Biol. Chem. 264:7564-9, 1989) and the segments between the transmembrane domains are relatively short, a feature consistent in other chemoattractant receptors (Boulay, F. et al. Biochemistry 30:2993-9, 1991; Boulay, F. et al. Biochemistry 29:11123-11133, 1990; Gerard, N. P. et al. Nature 349:614-7, 1991; Holmes, W. E. et al. Science 253:1278-80, 1991; Murphy, P.

M. et al. Science 253:1280-3, 1991). There are three potential glycosylation sites in the C-C CKR-1 (Figure 1), one in the N-terminus, one in the first cytoplasmic loop and the third in TM6. This latter site is unlikely to be glycosylated since it is predicted to be embedded in the cell membrane. Finally, there is a consensus sequence for a protein kinase C phosphorylation site, at position 192, but this position is predicted to be extracellular.

The deduced amino acid sequence of C-C CKR-1 was compared to other G-protein linked chemoattractant receptors (Figure 1). The IL8rA and IL8rB showed about 32% sequence identity to C-C CKR-1, while the C5a and fMLP receptors showed about 23% identity (Figure 1). A putative seven transmembrane spanning molecule, HUMSTSR, which has been recently been deposited in Genbank (accession #M99293) by Federsspiel, et al., was found to have about 31% identity with C-C CKR-1. The sequence of this molecule is identical to one of the putative receptors isolated in our first attempt at RT-PCR cloning described above. The ligand for HUMSTSR has not yet been identified. The second group of receptors which are as closely related to the C-C CKR-1 are the neuropeptide Y and the angiotensin II receptors (data not shown). Lastly, an open reading frame in the cytomegalovirus genome, designated US28, has about 33% identity with the C-C CKR-1 and about 60% identity in the N-terminal region before TM1 with C-C CKR-1.

### Example III

#### Northern and Southern analysis of C-C CKR-1

The expression of the C-C CKR-1 was assessed in a limited panel of hematopoietic cell lines using Northern blot analysis. Northern blot hybridization was performed as follows. Total RNA was isolated using the guanidinium isothiocyanate-CsCl procedure (Sambrook et al., 1989) or by the RNazol method as recommended by the supplier. Poly A<sup>+</sup> RNA was isolated using Dynabeads oligodT (DYNAL, Great Neck, NY) as recommended by the supplier. HL60 mRNA designated "HL60-C" in this report was obtained from Clontech (Palo Alto, CA). 20 µg of total RNA or 5 µg of

poly A+ RNA was fractionated on formaldehyde-agarose gels, blotted to a nitrocellulose membrane and hybridized with the C-C CKR-1 cDNA (Korneluk, R. G. et al. J. Biol. Chem. 261:8407-8413, 1986).

5           A single band of about 3 kb was detected in pre-monocytic cell lines, e.g. undifferentiated or differentiated U937 and HL60 cells (Figure 2), but not in a commercially available preparation of HL60 RNA (HL60-C, Figure 2). Lower levels of mRNA were also detected in B  
10 cell lines 1788 and Daudi, but little or no RNA was detected in K562 cells (Figure 2). The highest levels were detected in PMA-treated THP-1 cells, where a strong signal was obtained when 20 mg of total RNA was analyzed (Figure 2).

15           Southern blot hybridization was performed as follows. Human genomic DNA, obtained from Clontech (Palo Alto, CA), was restriction digested, blotted onto Genescreen® (Dupont) and hybridized (Neote, K., et al. J. Clin. Invest. 86:1524-31, 1990) with the C-C CKR-1 cDNA. The hybridization  
20 pattern indicated that the C-C CKR-1 gene could be intronless. Furthermore, it suggested the existence of a second related gene or possibly a pseudogene. Figures 3A and 3B represent low and high stringency washes of the same blot of human genomic DNA which has been digested with  
25 several restriction enzymes. Under high stringency conditions (Figure 3B), single restriction fragments hybridizing to the C-C CKR-1 cDNA were detected when genomic DNA was digested with BamHI, HindIII or SacI and, as predicted from the cDNA sequence, two fragments are  
30 detected in DNA digested with EcoRI and PstI. However, low stringency hybridization revealed the presence of additional bands that hybridized to the C-C CKR-1 cDNA e.g. an approximately 7 kb PstI fragment and an approximately  
35 bands disappeared when the blots were washed under high stringency conditions while the other bands remained unchanged between the two washes.

Example IVSignaling through the C-C CKR-1 in response toHuMIP-1 $\alpha$  and RANTES

MCP-1, RANTES, HuMIP-1 $\alpha$  and HuMIP-1 $\beta$  induce a rapid  
5 and transient increase in intracellular Ca<sup>++</sup> in human  
monocytes (Rollins, B. J. et al. Blood 78:1112-6, 1991;  
Sozzani, S. et al. J. Immunol. 147:2215-21, 1991). To  
determine if C-C CKR-1 was a functional C-C chemokine  
receptor, it was transiently expressed in human kidney  
10 cells and intracellular Ca<sup>++</sup> levels in response to  
different C-C chemokines were measured.

Recombinant RANTES was expressed in E. coli and  
purified as described by Kuna et al. (J. Immunol. 149:636-  
42, 1992b). HuMIP-1 $\alpha$  and HuMIP-1 $\beta$  was expressed in E.  
15 coli, and purified as described by Rot et al. (J. Exp.  
Med., in press). HuMIP-1 $\alpha$  was iodinated by the  
chloroglycoluril method (Fraker et al. Biochem. Biophys.  
Res. Comm. 80:849-857, 1978) to an initial specific  
activity of 472 Ci/mmol. The labeled HuMIP-1 $\alpha$  was purified  
20 by a combination of gel filtration and reversed-phase HPLC.  
The <sup>125</sup>I-labeled RANTES, specific activity 2200 Ci/mmol,  
was obtained from Dupont/NEN (Boston, MASS). Recombinant  
MCP-1 and murine MIP-1 $\alpha$  were obtained from Peprotech (Rocky  
Hill, NJ).

25 Human embryonic kidney 293 cells were transfected with  
10-20  $\mu$ g of plasmid DNA by the calcium-phosphate method as  
described (Schall, T. J. et al. Eur. J. Immunol. 22:1477-  
81, 1992) or the modified calcium phosphate method (Chen,  
C. et al. Mol. Cell. Biol. 7:2745-2752, 1987). The  
30 transfected cells were assayed after transient expression  
for 12-24 hours.

Intracellular Ca<sup>++</sup> measurements were done on the SLM  
8000C essentially as described (Naccache, P. H. et al. J.  
Immunol. 142:2438-44, 1989), with minor modifications:  
35 IND0-1-AM, was used at 20 mg/ml final concentration and 2-4  
X10<sup>6</sup> cells were used per assay.

A 100 nM dose of RANTES, HuMIP-1 $\alpha$ , HuMIP-1 $\beta$  and MCP-1 was initially used as a first approximation of the maximum physiologically-relevant concentration. When transfected cells, loaded with the calcium probe INDO-1-AM, were challenged with 100 nM of either HuMIP-1 $\alpha$  or RANTES, a rapid increase in intracellular Ca<sup>++</sup> was observed (Figure 4). Challenge with the same dose of MCP-1 or HuMIP-1 $\beta$  produced little detectable Ca<sup>++</sup> flux (data not shown). Control cells transfected with vector alone or vector containing C-C CKR-1 in the opposite (non-coding) orientation did not respond to any of the ligands (data not shown). The signaling responses were dose dependent, 10 nM of HuMIP-1 $\alpha$  and 100 nM of RANTES were sufficient to give a maximal or near maximal response (Figure 4A and 4B).

Rapid, successive exposure to the same ligand is known to desensitize the signaling capacity of G-protein linked receptors (Schild, H. O. (1973), "Receptor classification with special reference to  $\beta$ -adrenergic receptors," In Drug Receptors, H.P. Rang, ed. (University Press), pp. 29-36). In addition, desensitization can also occur when the two different agonists signal through the same receptor. HuMIP-1 $\alpha$  clearly blocks the ability of the C-C CKR-1 receptor to transmit a second Ca<sup>++</sup> signal when HuMIP-1 $\alpha$  is added to transfected cells twice in succession (Figure 5A). Similarly, RANTES blocks the response to a second challenge at the same concentration (Figure 5B). When HuMIP-1 $\alpha$  is added first, it blocks the response to RANTES, indicating that complete desensitization has occurred (Figure 5C). However, challenging C-C CKR-1 cDNA transfected cells with 250 nM RANTES did not prevent a subsequent Ca<sup>++</sup> flux by the addition of 100 nM HuMIP-1 $\alpha$  (Figure 5D), indicating that receptor desensitization had not occurred. However, the subsequent Ca<sup>++</sup> flux by HuMIP-1 $\alpha$  is reduced, from a intracellular Ca<sup>++</sup> change of about 70 nM (Figure 5A and C) to about 40 nM (Figure 5D), suggesting that a partial desensitization has occurred.

Example VBinding of HuMIP-1 $\alpha$  and RANTES to C-C CKR-1

In order to further investigate the interaction of HuMIP-1 $\alpha$  and RANTES to the cloned C-C CKR-1, direct binding experiments with  $^{125}\text{I}$ -labeled ligands were carried out.

Binding assays were performed as described previously (Horuk, R. et al. J. Biol. Chem. 262:16275-16278, 1987). Transfected cells ( $2 \times 10^6$  cells per ml) were incubated with radiolabeled ligands and varying concentrations of unlabeled ligands at 4 °C for 2 hours. The incubation was terminated by removing aliquots from the cell suspension and separating cells from buffer by centrifugation through a silicon/paraffin oil mixture as described previously (Robb, R. J. et al. J. Exp. Med. 160:1126-1146, 1984). Non-specific binding was determined in the presence of 1  $\mu\text{M}$  unlabeled ligand. Individual assay determinations, representative of at least three separate experiments were plotted. The binding data were curve fit with the computer program LIGAND (Munson, P. J. et al. Anal. Biochem. 107:220-239, 1980) modified for the IBM PC (McPherson, G. A. Comp. Prog. Biomed. 17:107-114, 1983) to determine the affinity ( $K_d$ ), number of sites, and nonspecific binding. The curves shown are the binding isotherms determined by LIGAND.

Thus, when 293 cells transiently expressing C-C CKR-1 were incubated with  $^{125}\text{I}$ -HuMIP-1 $\alpha$  and increasing concentrations of unlabeled HuMIP-1 $\alpha$ , displaceable binding of  $^{125}\text{I}$ -HuMIP-1 $\alpha$  to the C-C CKR-1 was observed (Figure 6A). Scatchard analysis showed a dissociation constant ( $K_d$ ) of  $5.1 \pm 0.3 \text{ nM}$  and about 130000 sites/cell. This  $K_d$  is within the range of that determined for HuMIP-1 $\alpha$  binding to human monocytes and suggests that C-C CKR-1 is at least one of the HuMIP-1 $\alpha$  receptors present on monocytes. Direct binding of RANTES to the C-C CKR-1 however could not be accomplished, i.e.,  $^{125}\text{I}$ -RANTES could not be displaced by unlabeled RANTES. Interestingly, as the amount of unlabeled RANTES was increased in the binding assay, a

concomitant increase of  $^{125}\text{I}$ -RANTES bound to cells was observed (data not shown). Since the C-C CKR-1 transduces a signal (mobilizes  $\text{Ca}^{++}$ ) in response to RANTES (Figure 4), and therefore, the ligand must be binding to the cloned  
5 receptor, the reason for the unusual binding profile observed for  $^{125}\text{I}$ -RANTES is not clear. Similar binding phenomenon are obtained if other target cells responding to RANTES are used. Interestingly,  $^{125}\text{I}$ -RANTES could be displaced by unlabeled HuMIP-1 $\alpha$  on 293 cells transiently  
10 expressing C-C CKR-1 (Figure 6B). Scatchard analysis of this heterologous displacement suggested a  $K_d$  of  $7.6 \pm 1.5 \text{ nM}$  (about 350000 sites/cell) and is consistent with the  $K_d$  of HuMIP-1 $\alpha$  binding data described above. These observations suggested to us initially that the C-C CKR-1 binds to  
15 HuMIP-1 $\alpha$  and RANTES and subsequently transduces a signal by increasing the intracellular  $\text{Ca}^{++}$  levels

#### Example VI

##### Displacement of HuMIP-1 $\alpha$ by Heterologous Chemokines

To further characterize the binding properties of the  
20 C-C CKR-1, and in particular to attempt to define a  $K_d$  for RANTES binding to the cloned C-C CKR-1, heterologous displacement of  $^{125}\text{I}$ -HuMIP-1 $\alpha$  was done with RANTES and also HuMIP-1 $\beta$ , MCP-1, IL-8, and murine MIP-1 $\alpha$ . Interestingly, all C-C chemokines (RANTES, MIP-1b and MCP-1) displaced  
25  $^{125}\text{I}$ -HuMIP-1 $\alpha$ , but the C-X-C chemokine IL-8 did not (Figure 7). The  $K_d$  for RANTES, HuMIP-1b and MCP-1, and murine MIP-1 $\alpha$  as determined from Scatchard analysis is  $468 \pm 280$ ,  $232 \pm 70.1$ ,  $122 \pm 39.3$ , and  $4.2 \pm 2.7 \text{ nM}$  respectively, and in each case about 250000-350000 sites/cell are present  
30 (Figure 7). These results revealed a broad ligand specificity of the C-C CKR-1, including some lack of species specificity in binding. More importantly, it suggested that the binding affinity between the ligand and the receptor does not predict the signaling efficacy  
35 resulting from that interaction, i.e. although HuMIP-1 $\beta$  and MCP-1 bind with a higher affinity than does RANTES, 100 nM of MIP-1 $\beta$  or MCP-1 do not transmit a signal via the cloned receptor, whereas RANTES does (Figure 4).

Example VIICalcium Mobilization in Response to HuMIP-1 $\beta$  and MCP-1

To determine the relevance of HuMIP-1 $\beta$  and MCP-1 binding to C-C CKR-1, C-C CKR-1 cDNA transfected 293 cells were challenged with high doses of HuMIP-1 $\beta$  and MCP-1 and intracellular Ca<sup>++</sup> levels were determined. A concentration of 1 mM of the recombinant MCP-1 had to be used to produce a Ca<sup>++</sup> flux. This Ca<sup>++</sup> flux, however, was only about 20% of the maximum response obtained by HuMIP-1 $\alpha$  or RANTES (Figure 8). Lower concentrations gave an almost undetectable flux (data not shown). Control experiments utilizing THP-1 cells and 50-100 nM of MCP-1 gave the expected strong Ca<sup>++</sup> fluxes (a transient increase of 200-300 nM Ca<sup>++</sup>, data not shown). Similarly, 250 nM of HuMIP-1 $\beta$  was needed to produce a Ca<sup>++</sup> flux in 293 cells transiently expressing C-C CKR-1 that was about 20% of the maximal signal obtained by HuMIP-1 $\alpha$  or RANTES (Figure 8). These results support the suggestion that the binding affinities HuMIP-1 $\beta$  and MCP-1 do not reflect their signaling capabilities.

20

Example VIIIBinding of HuMIP-1 $\alpha$  to 293 Cells Expressing the Protein Encoded by US28

Since the amino acid sequence of C-C CKR-1 is about 35% identical to an open reading frame in the human CMV genome designated as US28, we sought to determine if the protein encoded by this open reading frame would bind HuMIP-1 $\alpha$ . The US28 open reading frame was amplified from a stock of Towne strain cytomegalovirus (a kind gift of Dr. Philip Dormitzer) with primers flanking the coding region using PCR and the thermostable DNA polymerase Pfu. The PCR product was subcloned into the expression vector pRK5 and its identity confirmed by sequencing. Plasmid DNA containing US28 in the sense orientation and the opposite, antisense orientation was transfected into 293 cells. Transfected cells were then used to determine binding of radiolabeled HuMIP-1 $\alpha$ . 293 cells transfected with the US28 coding sequence in the sense



orientation bound  $^{125}\text{I}$ -HuMIP-1 $\alpha$ , whereas negligible binding was obtained in cells transfected with the antisense orientation (Figure 10). The radiolabeled HuMIP-1 $\alpha$  could also be displaced with 1  $\mu\text{M}$  of murine MIP-1 $\alpha$ , HuMIP-1 $\beta$ , RANTES, and

5 MCP-1, but not by the C-X-C chemokine, IL-8 (Figure 10). These results indicate that the protein encoded by US28 specifically binds C-C chemokines and not C-X-C chemokines.

## SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: GENENTECH, INC.
- (ii) TITLE OF INVENTION: CC-CHEMOKINE RECEPTOR
- (iii) NUMBER OF SEQUENCES: 11
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Genentech, Inc.
  - (B) STREET: 460 Point San Bruno Blvd
  - (C) CITY: South San Francisco
  - (D) STATE: California
  - (E) COUNTRY: USA
  - (F) ZIP: 94080
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: 5.25 inch, 360 Kb floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: patin (Genentech)
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Fitts, Renee A.
  - (B) REGISTRATION NUMBER: 35,136
  - (C) REFERENCE/DOCKET NUMBER: 806
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 415/225-1489
  - (B) TELEFAX: 415/952-9881
  - (C) TELEX: 910/371-7168

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 355 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Glu Thr Pro Asn Thr Thr Glu Asp Tyr Asp Thr Thr Thr Glu  
1 5 10 15

57

Phe	Asp	Tyr	Gly	Asp	Ala	Thr	Pro	Cys	Gln	Lys	Val	Asn	Glu	Arg		20	25	30
Ala	Phe	Gly	Ala	Gln	Leu	Leu	Pro	Pro	Leu	Tyr	Ser	Leu	Val	Phe		35	40	45
Val	Ile	Gly	Leu	Val	Gly	Asn	Ile	Leu	Val	Val	Leu	Val	Leu	Val		50	55	60
Gln	Tyr	Lys	Arg	Leu	Lys	Asn	Met	Thr	Ser	Ile	Tyr	Leu	Leu	Asn		65	70	75
Leu	Ala	Ile	Ser	Asp	Leu	Leu	Phe	Leu	Phe	Thr	Leu	Pro	Phe	Trp		80	85	90
Ile	Asp	Tyr	Lys	Leu	Lys	Asp	Asp	Trp	Val	Phe	Gly	Asp	Ala	Met		95	100	105
Cys	Lys	Ile	Leu	Ser	Gly	Phe	Tyr	Tyr	Thr	Gly	Leu	Tyr	Ser	Glu		110	115	120
Ile	Phe	Phe	Ile	Ile	Leu	Leu	Thr	Ile	Asp	Arg	Tyr	Leu	Ala	Ile		125	130	135
Val	His	Ala	Val	Phe	Ala	Leu	Arg	Ala	Arg	Thr	Val	Thr	Phe	Gly		140	145	150
Val	Ile	Thr	Ser	Ile	Ile	Ile	Trp	Ala	Leu	Ala	Ile	Leu	Ala	Ser		155	160	165
Met	Pro	Gly	Leu	Tyr	Phe	Ser	Lys	Thr	Gln	Trp	Glu	Phe	Thr	His		170	175	180
His	Thr	Cys	Ser	Leu	His	Phe	Pro	His	Glu	Ser	Leu	Arg	Glu	Trp		185	190	195
Lys	Leu	Phe	Gln	Ala	Leu	Lys	Leu	Asn	Leu	Phe	Gly	Leu	Val	Leu		200	205	210
Pro	Leu	Leu	Val	Met	Ile	Ile	Cys	Tyr	Thr	Gly	Ile	Ile	Lys	Ile		215	220	225
Leu	Leu	Arg	Arg	Pro	Asn	Glu	Lys	Lys	Ser	Lys	Ala	Val	Arg	Leu		230	235	240
Ile	Phe	Val	Ile	Met	Ile	Ile	Phe	Phe	Leu	Phe	Trp	Thr	Pro	Tyr		245	250	255
Asn	Leu	Thr	Ile	Leu	Ile	Ser	Val	Phe	Gln	Asp	Phe	Leu	Phe	Thr		260	265	270
His	Glu	Cys	Glu	Gln	Ser	Arg	His	Leu	Asp	Leu	Ala	Val	Gln	Val		275	280	285
Thr	Glu	Val	Ile	Ala	Tyr	Thr	His	Cys	Cys	Val	Asn	Pro	Val	Ile		290	295	300

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Tyr Ala Phe Val Gly Glu Arg Phe Arg Lys Tyr Leu Arg Gln Leu  
 305 310 315  
 Phe His Arg Arg Val Ala Val His Leu Val Lys Trp Leu Pro Phe  
 320 325 330  
 Leu Ser Val Asp Arg Leu Glu Arg Val Ser Ser Thr Ser Pro Ser  
 335 340 345  
 Thr Gly Glu His Glu Leu Ser Ala Gly Phe  
 350 355

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 352 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Glu Gly Ile Ser Ile Tyr Thr Ser Asp Asn Tyr Thr Glu Glu  
 1 5 10 15  
 Met Gly Ser Gly Asp Tyr Asp Ser Met Lys Glu Pro Cys Phe Arg  
 20 25 30  
 Glu Glu Asn Ala Asn Phe Asn Lys Ile Phe Leu Pro Thr Ile Tyr  
 35 40 45  
 Ser Ile Ile Phe Leu Thr Gly Ile Val Gly Asn Gly Leu Val Ile  
 50 55 60  
 Leu Val Met Gly Tyr Gln Lys Lys Leu Arg Ser Met Thr Asp Lys  
 65 70 75  
 Tyr Arg Leu His Leu Ser Val Ala Asp Leu Leu Phe Val Ile Thr  
 80 85 90  
 Leu Pro Phe Trp Ala Val Asp Ala Val Ala Asn Trp Tyr Phe Gly  
 95 100 105  
 Asn Phe Leu Cys Lys Ala Val His Val Ile Tyr Thr Val Asn Leu  
 110 115 120  
 Tyr Ser Ser Val Leu Ile Leu Ala Phe Ile Ser Leu Asp Arg Tyr  
 125 130 135  
 Leu Ala Ile Val His Ala Thr Asn Ser Gln Arg Pro Arg Lys Leu  
 140 145 150  
 Leu Ala Glu Lys Val Val Tyr Val Gly Val Trp Ile Pro Ala Leu  
 155 160 165  
 Leu Leu Thr Ile Pro Asp Phe Ile Phe Ala Asn Val Ser Glu Ala  
 170 175 180

Asp	Asp	Arg	Tyr	Ile	Cys	Asp	Arg	Phe	Tyr	Pro	Asn	Asp	Leu	Trp	185	190	195
Val	Val	Val	Phe	Gln	Phe	Gln	His	Ile	Met	Val	Gly	Leu	Ile	Leu	200	205	210
Pro	Gly	Ile	Val	Ile	Leu	Ser	Cys	Tyr	Cys	Ile	Ile	Ile	Ser	Lys	215	220	225
Leu	Ser	His	Ser	Lys	Gly	His	Gln	Lys	Arg	Lys	Ala	Leu	Lys	Thr	230	235	240
Thr	Val	Ile	Leu	Ile	Leu	Ala	Phe	Phe	Ala	Cys	Trp	Leu	Pro	Tyr	245	250	255
Tyr	Ile	Gly	Ile	Ser	Ile	Asp	Ser	Phe	Ile	Leu	Leu	Glu	Ile	Ile	260	265	270
Lys	Gln	Gly	Cys	Glu	Phe	Glu	Asn	Thr	Val	His	Lys	Trp	Ile	Ser	275	280	285
Ile	Thr	Glu	Ala	Leu	Ala	Phe	Phe	His	Cys	Cys	Leu	Asn	Pro	Ile	290	295	300
Leu	Tyr	Ala	Phe	Leu	Gly	Ala	Lys	Phe	Lys	Thr	Ser	Ala	Gln	His	305	310	315
Ala	Leu	Thr	Ser	Val	Ser	Arg	Gly	Ser	Ser	Leu	Lys	Ile	Leu	Ser	320	325	330
Lys	Gly	Lys	Arg	Gly	Gly	His	Ser	Ser	Val	Ser	Thr	Glu	Ser	Glu	335	340	345
Ser	Ser	Ser	Phe	His	Ser	Ser									350	352	

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 350 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met	Ser	Asn	Ile	Thr	Asp	Pro	Gln	Met	Trp	Asp	Phe	Asp	Asp	Leu	1	5	10	15
Asn	Phe	Thr	Gly	Met	Pro	Pro	Ala	Asp	Glu	Asp	Tyr	Ser	Pro	Cys	20	25	30	
Met	Leu	Glu	Thr	Glu	Thr	Leu	Asn	Lys	Tyr	Val	Val	Ile	Ile	Ala	35	40	45	
Tyr	Ala	Leu	Val	Phe	Leu	Leu	Ser	Leu	Leu	Gly	Asn	Ser	Leu	Val	50	55	60	

60

Met	Leu	Val	Ile	Leu	Tyr	Ser	Arg	Val	Gly	Arg	Ser	Val	Thr	Asp	65	70	75
Val	Tyr	Leu	Leu	Asn	Leu	Ala	Leu	Ala	Asp	Leu	Leu	Phe	Ala	Leu	80	85	90
Thr	Leu	Pro	Ile	Trp	Ala	Ala	Ser	Lys	Val	Asn	Gly	Trp	Ile	Phe	95	100	105
Gly	Thr	Phe	Leu	Cys	Lys	Val	Val	Ser	Leu	Leu	Lys	Glu	Val	Asn	110	115	120
Phe	Tyr	Ser	Gly	Ile	Leu	Leu	Leu	Ala	Cys	Ile	Ser	Val	Asp	Arg	125	130	135
Tyr	Leu	Ala	Ile	Val	His	Ala	Thr	Arg	Thr	Leu	Thr	Gln	Lys	Arg	140	145	150
His	Leu	Val	Lys	Phe	Val	Cys	Leu	Gly	Cys	Trp	Gly	Leu	Ser	Met	155	160	165
Asn	Leu	Ser	Leu	Pro	Phe	Phe	Leu	Phe	Arg	Gln	Ala	Tyr	His	Pro	170	175	180
Asn	Asn	Ser	Ser	Pro	Val	Cys	Tyr	Glu	Val	Leu	Gly	Asn	Asp	Thr	185	190	195
Ala	Lys	Trp	Arg	Met	Val	Leu	Arg	Ile	Leu	Pro	His	Thr	Phe	Gly	200	205	210
Phe	Ile	Val	Pro	Leu	Phe	Val	Met	Leu	Phe	Cys	Tyr	Gly	Phe	Thr	215	220	225
Leu	Arg	Thr	Leu	Phe	Lys	Ala	His	Met	Gly	Gln	Lys	His	Arg	Ala	230	235	240
Met	Arg	Val	Ile	Phe	Ala	Val	Val	Leu	Ile	Phe	Leu	Leu	Cys	Trp	245	250	255
Leu	Pro	Tyr	Asn	Leu	Val	Leu	Leu	Ala	Asp	Thr	Leu	Met	Arg	Thr	260	265	270
Gln	Val	Ile	Gln	Glu	Thr	Cys	Glu	Arg	Arg	Asn	Asn	Ile	Gly	Arg	275	280	285
Ala	Leu	Asp	Ala	Thr	Glu	Ile	Leu	Gly	Phe	Leu	His	Ser	Cys	Leu	290	295	300
Asn	Pro	Ile	Ile	Tyr	Ala	Phe	Ile	Gly	Gln	Asn	Phe	Arg	His	Gly	305	310	315
Phe	Leu	Lys	Ile	Leu	Ala	Met	His	Gly	Leu	Val	Ser	Lys	Glu	Phe	320	325	330
Leu	Ala	Arg	His	Arg	Val	Thr	Ser	Tyr	Thr	Ser	Ser	Ser	Val	Asn	335	340	345

Val Ser Ser Asn Leu  
350

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 355 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met	Glu	Ser	Asp	Ser	Phe	Glu	Asp	Phe	Trp	Lys	Gly	Glu	Asp	Leu	1	5	10	15
Ser	Asn	Tyr	Ser	Tyr	Ser	Ser	Thr	Leu	Pro	Pro	Phe	Leu	Leu	Asp	20	25	30	
Ala	Ala	Pro	Cys	Glu	Pro	Glu	Ser	Leu	Glu	Ile	Asn	Lys	Tyr	Phe	35	40	45	
Val	Val	Ile	Ile	Tyr	Ala	Leu	Val	Phe	Leu	Leu	Ser	Leu	Leu	Gly	50	55	60	
Asn	Ser	Leu	Val	Met	Leu	Val	Ile	Leu	Tyr	Ser	Arg	Val	Gly	Arg	65	70	75	
Ser	Val	Thr	Asp	Val	Tyr	Leu	Leu	Asn	Leu	Ala	Leu	Ala	Asp	Leu	80	85	90	
Leu	Phe	Ala	Leu	Thr	Leu	Pro	Ile	Trp	Ala	Ala	Ser	Lys	Val	Asn	95	100	105	
Gly	Trp	Ile	Phe	Gly	Thr	Phe	Leu	Cys	Lys	Val	Val	Ser	Leu	Leu	110	115	120	
Lys	Glu	Val	Asn	Phe	Tyr	Ser	Gly	Ile	Leu	Leu	Leu	Ala	Cys	Ile	125	130	135	
Ser	Val	Asp	Arg	Tyr	Leu	Ala	Ile	Val	His	Ala	Thr	Arg	Thr	Leu	140	145	150	
Thr	Gln	Lys	Arg	Tyr	Leu	Val	Lys	Phe	Ile	Cys	Leu	Ser	Ile	Trp	155	160	165	
Gly	Leu	Ser	Leu	Leu	Leu	Ala	Leu	Pro	Val	Leu	Leu	Phe	Arg	Arg	170	175	180	
Thr	Val	Tyr	Ser	Ser	Asn	Val	Ser	Pro	Ala	Cys	Tyr	Glu	Asp	Met	185	190	195	
Gly	Asn	Asn	Thr	Ala	Asn	Trp	Arg	Met	Leu	Leu	Arg	Ile	Leu	Pro	200	205	210	
Gln	Ser	Phe	Gly	Phe	Ile	Val	Pro	Leu	Leu	Ile	Met	Leu	Phe	Cys	215	220	225	

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Tyr	Gly	Phe	Thr	Leu	Arg	Thr	Leu	Phe	Lys	Ala	His	Met	Gly	Gln
				230					235					240
Lys	His	Arg	Ala	Met	Arg	Val	Ile	Phe	Ala	Val	Val	Leu	Ile	Phe
				245					250					255
Leu	Leu	Cys	Trp	Leu	Pro	Tyr	Asn	Leu	Val	Leu	Leu	Ala	Asp	Thr
				260					265					270
Leu	Met	Arg	Thr	Gln	Val	Ile	Gln	Glu	Thr	Cys	Glu	Arg	Arg	Asn
				275					280					285
His	Ile	Asp	Arg	Ala	Leu	Asp	Ala	Thr	Glu	Ile	Leu	Gly	Ile	Leu
				290					295					300
His	Ser	Cys	Leu	Asn	Pro	Leu	Ile	Tyr	Ala	Phe	Ile	Gly	Gln	Lys
				305					310					315
Phe	Arg	His	Gly	Leu	Leu	Lys	Ile	Leu	Ala	Ile	His	Gly	Leu	Ile
				320					325					330
Ser	Lys	Asp	Ser	Leu	Pro	Lys	Asp	Ser	Arg	Pro	Ser	Phe	Val	Gly
				335					340					345
Ser	Ser	Ser	Gly	His	Thr	Ser	Thr	Thr	Leu					
				350					355					

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 350 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met	Asn	Ser	Phe	Asn	Tyr	Thr	Thr	Pro	Asp	Tyr	Gly	His	Tyr	Asp
1				5					10					15
Asp	Lys	Asp	Thr	Leu	Asp	Leu	Asn	Thr	Pro	Val	Asp	Lys	Thr	Ser
				20					25					30
Asn	Thr	Leu	Arg	Val	Pro	Asp	Ile	Leu	Ala	Leu	Val	Ile	Phe	Ala
				35					40					45
Val	Val	Phe	Leu	Val	Gly	Val	Leu	Gly	Asn	Ala	Leu	Val	Val	Trp
				50					55					60
Val	Thr	Ala	Phe	Glu	Ala	Lys	Arg	Thr	Ile	Asn	Ala	Ile	Trp	Phe
				65					70					75
Leu	Asn	Leu	Ala	Val	Ala	Asp	Phe	Leu	Ser	Cys	Leu	Ala	Leu	Pro
				80					85					90
Ile	Leu	Phe	Thr	Ser	Ile	Val	Gln	His	His	His	Trp	Pro	Phe	Gly
				95					100					105



Gly	Ala	Ala	Cys	Ser	Ile	Leu	Pro	Ser	Leu	Ile	Leu	Leu	Asn	Met	110	115	120
Tyr	Ala	Ser	Ile	Leu	Leu	Leu	Ala	Thr	Ile	Ser	Ala	Asp	Arg	Phe	125	130	135
Leu	Leu	Val	Phe	Lys	Pro	Ile	Trp	Cys	Gln	Asn	Phe	Arg	Gly	Ala	140	145	150
Gly	Leu	Ala	Trp	Ile	Ala	Cys	Ala	Val	Ala	Trp	Gly	Leu	Ala	Leu	155	160	165
Leu	Leu	Thr	Ile	Pro	Ser	Phe	Leu	Tyr	Arg	Val	Val	Arg	Glu	Glu	170	175	180
Tyr	Phe	Pro	Pro	Lys	Val	Leu	Cys	Gly	Val	Asp	Tyr	Ser	His	Asp	185	190	195
Lys	Arg	Arg	Glu	Arg	Ala	Val	Ala	Ile	Val	Arg	Leu	Val	Leu	Gly	200	205	210
Phe	Leu	Trp	Pro	Leu	Leu	Thr	Leu	Thr	Ile	Cys	Tyr	Thr	Phe	Ile	215	220	225
Leu	Leu	Arg	Thr	Trp	Ser	Arg	Arg	Ala	Thr	Arg	Ser	Thr	Lys	Thr	230	235	240
Leu	Lys	Val	Val	Val	Ala	Val	Val	Ala	Ser	Phe	Phe	Ile	Phe	Trp	245	250	255
Leu	Pro	Tyr	Gln	Val	Thr	Gly	Ile	Met	Met	Ser	Phe	Leu	Glu	Pro	260	265	270
Ser	Ser	Pro	Thr	Phe	Leu	Leu	Leu	Asn	Lys	Leu	Asp	Ser	Leu	Cys	275	280	285
Val	Ser	Phe	Ala	Tyr	Ile	Asn	Cys	Cys	Ile	Asn	Pro	Ile	Ile	Tyr	290	295	300
Val	Val	Ala	Gly	Gln	Gly	Phe	Gln	Gly	Arg	Leu	Arg	Lys	Ser	Leu	305	310	315
Pro	Ser	Leu	Leu	Arg	Asn	Val	Leu	Thr	Glu	Glu	Ser	Val	Val	Arg	320	325	330
Glu	Ser	Lys	Ser	Phe	Thr	Arg	Ser	Thr	Val	Asp	Thr	Met	Ala	Gln	335	340	345
Lys	Thr	Gln	Ala	Val											350		

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 350 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met	Glu	Thr	Asn	Ser	Ser	Leu	Pro	Thr	Asn	Ile	Ser	Gly	Gly	Thr	1	5	10	15
Pro	Ala	Val	Ser	Ala	Gly	Tyr	Leu	Phe	Leu	Asp	Ile	Ile	Thr	Tyr	20	25	30	
Leu	Val	Phe	Ala	Val	Thr	Phe	Val	Leu	Gly	Val	Leu	Gly	Asn	Gly	35	40	45	
Leu	Val	Ile	Trp	Val	Ala	Gly	Phe	Arg	Met	Thr	His	Thr	Val	Thr	50	55	60	
Thr	Ile	Ser	Tyr	Leu	Asn	Leu	Ala	Val	Ala	Asp	Phe	Cys	Phe	Thr	65	70	75	
Ser	Thr	Leu	Pro	Phe	Phe	Met	Val	Arg	Lys	Ala	Met	Gly	Gly	His	80	85	90	
Trp	Pro	Phe	Gly	Trp	Phe	Leu	Cys	Lys	Phe	Val	Phe	Thr	Ile	Val	95	100	105	
Asp	Ile	Asn	Leu	Phe	Gly	Ser	Val	Phe	Leu	Ile	Ala	Leu	Ile	Ala	110	115	120	
Leu	Asp	Arg	Cys	Val	Cys	Val	Leu	His	Pro	Val	Trp	Thr	Gln	Asn	125	130	135	
His	Arg	Thr	Val	Ser	Leu	Ala	Lys	Lys	Val	Ile	Ile	Gly	Pro	Trp	140	145	150	
Val	Met	Ala	Leu	Leu	Leu	Thr	Leu	Pro	Val	Ile	Ile	Arg	Val	Thr	155	160	165	
Thr	Val	Pro	Gly	Lys	Thr	Gly	Thr	Val	Ala	Cys	Thr	Phe	Asn	Phe	170	175	180	
Ser	Pro	Trp	Thr	Asn	Asp	Pro	Lys	Glu	Arg	Ile	Asn	Val	Ala	Val	185	190	195	
Ala	Met	Leu	Thr	Val	Arg	Gly	Ile	Ile	Arg	Phe	Ile	Ile	Gly	Phe	200	205	210	
Ser	Ala	Pro	Met	Ser	Ile	Val	Ala	Val	Ser	Tyr	Gly	Leu	Ile	Ala	215	220	225	
Thr	Lys	Ile	His	Lys	Gln	Gly	Leu	Ile	Lys	Ser	Ser	Arg	Pro	Leu	230	235	240	

65

Arg	Val	Leu	Ser	Phe	Val	Ala	Ala	Ala	Phe	Phe	Leu	Cys	Trp	Ser	245	250	255
Pro	Tyr	Gln	Val	Val	Ala	Leu	Ile	Ala	Thr	Val	Arg	Ile	Arg	Glu	260	265	270
Leu	Leu	Gln	Gly	Met	Tyr	Lys	Glu	Ile	Gly	Ile	Ala	Val	Asp	Val	275	280	285
Thr	Ser	Ala	Leu	Ala	Phe	Phe	Asn	Ser	Cys	Leu	Asn	Pro	Met	Leu	290	295	300
Tyr	Val	Phe	Met	Gly	Gln	Asp	Phe	Arg	Glu	Arg	Leu	Ile	His	Ala	305	310	315
Leu	Pro	Ala	Ser	Leu	Glu	Arg	Ala	Leu	Thr	Glu	Asp	Ser	Thr	Gln	320	325	330
Thr	Ser	Asp	Thr	Ala	Thr	Asn	Ser	Thr	Leu	Pro	Ser	Ala	Glu	Val	335	340	345
Glu	Leu	Gln	Ala	Lys											350		

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 323 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met	Thr	Pro	Thr	Thr	Thr	Thr	Ala	Glu	Leu	Thr	Thr	Glu	Phe	Asp	1	5	10	15
Tyr	Asp	Glu	Asp	Ala	Thr	Pro	Cys	Val	Phe	Thr	Asp	Val	Leu	Asn	20	25	30	
Gln	Ser	Lys	Pro	Val	Thr	Leu	Phe	Leu	Tyr	Gly	Val	Val	Phe	Leu	35	40	45	
Phe	Gly	Ser	Ile	Gly	Asn	Phe	Leu	Val	Ile	Phe	Thr	Ile	Thr	Trp	50	55	60	
Arg	Arg	Arg	Ile	Gln	Cys	Ser	Gly	Asp	Val	Tyr	Phe	Ile	Asn	Leu	65	70	75	
Ala	Ala	Ala	Asp	Leu	Leu	Phe	Val	Cys	Thr	Leu	Pro	Leu	Trp	Met	80	85	90	
Gln	Tyr	Leu	Leu	Asp	His	Asn	Ser	Leu	Ala	Ser	Val	Pro	Cys	Thr	95	100	105	
Leu	Leu	Thr	Ala	Cys	Phe	Tyr	Val	Ala	Met	Phe	Ala	Ser	Leu	Cys	110	115	120	

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Phe	Ile	Thr	Glu	Ile	Ala	Leu	Asp	Arg	Tyr	Tyr	Ala	Ile	Val	Tyr	125	130	135
Met	Arg	Tyr	Arg	Pro	Val	Lys	Gln	Ala	Cys	Leu	Phe	Ser	Ile	Phe	140	145	150
Trp	Trp	Ile	Phe	Ala	Val	Ile	Ile	Ala	Ile	Pro	His	Phe	Met	Val	155	160	165
Val	Thr	Lys	Lys	Asp	Asn	Gln	Cys	Met	Thr	Asp	Tyr	Asp	Tyr	Leu	170	175	180
Glu	Val	Ser	Tyr	Pro	Ile	Ile	Leu	Asn	Val	Glu	Leu	Met	Leu	Gly	185	190	195
Ala	Phe	Val	Ile	Pro	Leu	Ser	Val	Ile	Ser	Tyr	Cys	Tyr	Tyr	Arg	200	205	210
Ile	Ser	Arg	Ile	Val	Ala	Val	Ser	Gln	Ser	Arg	His	Lys	Gly	Arg	215	220	225
Ile	Val	Arg	Val	Leu	Ile	Ala	Val	Val	Leu	Val	Phe	Ile	Ile	Phe	230	235	240
Trp	Leu	Pro	Tyr	His	Leu	Thr	Leu	Phe	Val	Asp	Thr	Leu	Lys	Leu	245	250	255
Leu	Lys	Trp	Ile	Ser	Ser	Ser	Cys	Glu	Phe	Glu	Arg	Ser	Leu	Lys	260	265	270
Arg	Ala	Leu	Ile	Leu	Thr	Glu	Ser	Leu	Ala	Phe	Cys	His	Cys	Cys	275	280	285
Leu	Asn	Pro	Leu	Leu	Tyr	Val	Phe	Val	Gly	Thr	Lys	Phe	Arg	Lys	290	295	300
Asn	Tyr	Thr	Val	Cys	Trp	Pro	Ser	Phe	Ala	Ser	Asp	Ser	Phe	Pro	305	310	315
Ala	Met	Tyr	Pro	Gly	Thr	Thr	Ala								320	323	

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1495 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATG	GAA	ACT	CCA	AAC	ACC	ACA	GAG	GAC	TAT	GAC	ACG	36
Met	Glu	Thr	Pro	Asn	Thr	Thr	Glu	Asp	Tyr	Asp	Thr	
1				5					10			

67

ACC ACA GAG TTT GAC TAT GGG GAT GCA ACT CCG TGC CAG 75  
 Thr Thr Glu Phe Asp Tyr Gly Asp Ala Thr Pro Cys Gln  
 15 20 25

AAG GTG AAC GAG AGG GCC TTT GGG GCC CAA CTG CTG CCC 114  
 Lys Val Asn Glu Arg Ala Phe Gly Ala Gln Leu Leu Pro  
 30 35

CCT CTG TAC TCC TTG GTA TTT GTC ATT GGC CTG GTT GGA 153  
 Pro Leu Tyr Ser Leu Val Phe Val Ile Gly Leu Val Gly  
 40 45 50

AAC ATC CTG GTG GTC CTG GTC CTT GTG CAA TAC AAG AGG 192  
 Asn Ile Leu Val Val Leu Val Leu Val Gln Tyr Lys Arg  
 55 60

CTA AAA AAC ATG ACC AGC ATC TAC CTC CTG AAC CTG GCC 231  
 Leu Lys Asn Met Thr Ser Ile Tyr Leu Leu Asn Leu Ala  
 65 70 75

ATT TCT GAC CTG CTC TTC CTG TTC ACG CTT CCC TTC TGG 270  
 Ile Ser Asp Leu Leu Phe Leu Phe Thr Leu Pro Phe Trp  
 80 85 90

ATC GAC TAC AAG TTG AAG GAT GAC TGG GTT TTT GGT GAT 309  
 Ile Asp Tyr Lys Leu Lys Asp Asp Trp Val Phe Gly Asp  
 95 100

GCC ATG TGT AAG ATC CTC TCT GGG TTT TAT TAC ACA GGC 348  
 Ala Met Cys Lys Ile Leu Ser Gly Phe Tyr Tyr Thr Gly  
 105 110 115

TTG TAC AGC GAG ATC TTT TTC ATC ATC CTG CTG ACG ATT 387  
 Leu Tyr Ser Glu Ile Phe Phe Ile Ile Leu Leu Thr Ile  
 120 125

GAC AGG TAC CTG GCC ATC GTC CAC GCC GTG TTT GCC TTG 426  
 Asp Arg Tyr Leu Ala Ile Val His Ala Val Phe Ala Leu  
 130 135 140

CGG GCA CGG ACC GTC ACT TTT GGT GTC ATC ACC AGC ATC 465  
 Arg Ala Arg Thr Val Thr Phe Gly Val Ile Thr Ser Ile  
 145 150 155

ATC ATT TGG GCC CTG GCC ATC TTG GCT TCC ATG CCA GGC 504  
 Ile Ile Trp Ala Leu Ala Ile Leu Ala Ser Met Pro Gly  
 160 165

TTA TAC TTT TCC AAG ACC CAA TGG GAA TTC ACT CAC CAC 543  
 Leu Tyr Phe Ser Lys Thr Gln Trp Glu Phe Thr His His  
 170 175 180

ACC TGC AGC CTT CAC TTT CCT CAC GAA AGC CTA CGA GAG 582  
 Thr Cys Ser Leu His Phe Pro His Glu Ser Leu Arg Glu  
 185 190

68

TGG AAG CTG TTT CAG GCT CTG AAA CTG AAC CTC TTT GGG 621  
 Trp Lys Leu Phe Gln Ala Leu Lys Leu Asn Leu Phe Gly  
 195 200 205

CTG GTA TTG CCT TTG TTG GTC ATG ATC ATC TGC TAC ACA 660  
 Leu Val Leu Pro Leu Leu Val Met Ile Ile Cys Tyr Thr  
 210 215 220

GGG ATT ATA AAG ATT CTG CTA AGA CGA CCA AAT GAG AAG 699  
 Gly Ile Ile Lys Ile Leu Leu Arg Arg Pro Asn Glu Lys  
 225 230

AAA TCC AAA GCT GTC CGT TTG ATT TTT GTC ATC ATG ATC 738  
 Lys Ser Lys Ala Val Arg Leu Ile Phe Val Ile Met Ile  
 235 240 245

ATC TTT TTT CTC TTT TGG ACC CCC TAC AAT TTG ACT ATA 777  
 Ile Phe Phe Leu Phe Trp Thr Pro Tyr Asn Leu Thr Ile  
 250 255

CTT ATT TCT GTT TTC CAA GAC TTC CTG TTC ACC CAT GAG 816  
 Leu Ile Ser Val Phe Gln Asp Phe Leu Phe Thr His Glu  
 260 265 270

TGT GAG CAG AGC AGA CAT TTG GAC CTG GCT GTG CAA GTG 855  
 Cys Glu Gln Ser Arg His Leu Asp Leu Ala Val Gln Val  
 275 280 285

ACG GAG GTG ATC GCC TAC ACG CAC TGC TGT GTC AAC CCA 894  
 Thr Glu Val Ile Ala Tyr Thr His Cys Cys Val Asn Pro  
 290 295

GTG ATC TAC GCC TTC GTT GGT GAG AGG TTC CGG AAG TAC 933  
 Val Ile Tyr Ala Phe Val Gly Glu Arg Phe Arg Lys Tyr  
 300 305 310

CTG CGG CAG TTG TTC CAC AGG CGT GTG GCT GTG CAC CTG 972  
 Leu Arg Gln Leu Phe His Arg Arg Val Ala Val His Leu  
 315 320

GTT AAA TGG CTC CCC TTC CTC TCC GTG GAC AGG CTG GAG 1011  
 Val Lys Trp Leu Pro Phe Leu Ser Val Asp Arg Leu Glu  
 325 330 335

AGG GTC AGC TCC ACA TCT CCC TCC ACA GGG GAG CAT GAA 1050  
 Arg Val Ser Ser Thr Ser Pro Ser Thr Gly Glu His Glu  
 340 345 350

CTC TCT GCT GGG TTC TGA CT CAG ACC ATAG GAG GCC AACC 1090  
 Leu Ser Ala Gly Phe  
 355

CAAAATAAGC AGGCGTGACC TGCCAGGCAC ACTGACCAGC AGCCTGGCTC 1140

TCCCAGCCAG GTTCTGACTC TTGGCACAGC ATGGAGTCCG CCTCTTGAT 1190

AGAGAGGAAT GTAATGGTGG CCTGGGGCTT CTGAGGCTTC TGGGCTTGAG 1240

TCTTTTCCAT GAACTTCTCC CCTGGTAGAA AAGAAGATGA ATGAGCAAAA 1290

CCAAATATTC CAGAGACTGG GACTAAGTGT ACCAGAGAAG GGCTTGGACT 1340

CAAGCAAGAT TTCAGATTTG TGACCATTAG CATTTGTCAA CAAAGTCACC 1390

CACTTCCCAC TATTGCTTGC ACAAACCAAT TAAACCCAGT AGTGGTGACT 1440

GTGGGCTCCA TTCAAAGTGA GCTCCTAAGC CATGGGAGAC ACTGATGTAT 1490

GAGGA 1495

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Leu Asn Leu Ala Xaa Ala Asp Xaa Xaa  
1 5 9

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Asn Pro Xaa Xaa Tyr Xaa Xaa Xaa Gly Gln  
1 5 10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

70

Asp Arg Tyr Leu Ala Ile Val His Ala  
1 5 9



## WE CLAIM:

1. An isolated C-C CKR-1 polypeptide.
2. A composition comprising the C-C CKR-1 of claim 1, which composition is free of a contaminating polypeptide of the animal species that is the source of C-C CKR-1 polypeptide.
3. An antibody that is capable of binding C-C CKR-1 polypeptide of claim 1 that does not cross-react with a known chemokine receptor.
4. An isolated nucleic acid molecule encoding a C-C CKR-1 polypeptide.
5. The nucleic acid molecule of claim 4 which is DNA and contains greater than about 25 bases.
6. The nucleic acid molecule of claim 4 further comprising a promoter operably linked to the nucleic acid sequence.
7. The nucleic acid molecule of claim 6, wherein the promoter is heterologous to the C-C CKR-1.
8. An expression vector comprising the nucleic acid sequence of claim 4 operably linked to control sequences recognized by a host cell transformed with the vector.

9. A host cell transformed with the vector of claim 8.
10. A method of determining the presence of a C-C CKR-1 nucleic acid, comprising hybridizing nucleic acid encoding or complementary to C-C CKR-1 nucleic acid to a test sample nucleic acid and determining the presence of C-C CKR-1 nucleic acid.
11. A method of amplifying a nucleic acid test sample comprising priming a nucleic acid polymerase reaction with nucleic acid encoding or complementary to a C-C CKR-1 nucleic acid.
12. A composition comprising the C-C CKR-1 polypeptide of claim 1 and a pharmaceutically acceptable carrier.
13. A composition comprising the antibody of claim 3 and a pharmaceutically acceptable carrier.
14. An isolated polypeptide comprising the C-C CKR-1 polypeptide of claim 1 fused to a polypeptide heterologous to the C-C CKR-1.
15. A DNA isolate able to hybridize under stringent conditions to a nucleotide sequence encoding C-C CKR-1 having the amino acid sequence shown in Figure 1 and encoding a polypeptide having C-C CKR-1 biological activity.
16. The DNA isolate of claim 15 wherein the DNA isolate

is free of genomic DNA which encodes another polypeptide from the source of the DNA isolate.

17. The DNA isolate of claim 15, wherein the DNA encodes a polypeptide having the amino acid sequence shown in Figure 1.

18. The DNA isolate of claim 15, wherein the DNA encodes a polypeptide having at least about 50% sequence identity over the length of the polypeptide with that of the C-C CKR-1 amino acid sequence shown in Figure 1.

19. The DNA isolate of claim 15, wherein the DNA encodes a polypeptide having at least about 60% sequence identity with that of the C-C CKR-1 amino acid sequence shown in Figure 1.

20. The DNA isolate of claim 15 that is a cDNA sequence.

21. The DNA isolate of claim 15 that is a genomic sequence.

22. A method for identifying a C-C chemokine receptor comprising

(a) designing a PCR primer corresponding to a transmembrane region of a seven-transmembrane spanning protein;

(b) priming a PCR reaction with a cDNA substrate from a hematopoietic cell type known to respond to a C-C

chemokine;

(c) recovering PCR products from step (b).

23. The method of claim 22, wherein the primer comprises a set of degenerate oligonucleotides encoding the sequence

LNLA(L/V)AD(L/F)(L/G).

24. The method of claim 22, wherein the primer comprises a set of degenerate oligonucleotides encoding the sequence

NP(I/M)(I/L)Y(A/V)(F/V)(I/M/A)GQ.

25. The method of claim 22, wherein the primer comprises a set of degenerate oligonucleotides encoding the sequence

DRYLAIVHA.

26. The method of claim 22, wherein the cell type is cultured human monocytes.

27. A method comprising transforming a host cell with DNA encoding C-C CKR-1, culturing the host cell to express the receptor on its surface, harvesting the cells, contacting the cells with a C-C chemokine variant, and determining the biological activity of the variant on the receptor.

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C-CKR-I  
 HUMSTR  
 IL8rA  
 IL8rB  
 C5a  
 fMLP  
 hcmv-us28

1 ME T P N T T E D Y . . . . . D T T E F D Y G D . A T P C Q K V N E R A F G A Q L L P P L  
 1 ME G I S I Y T S D N . . . . . Y T E E M G S G D Y D S M K E P C F R E E N A N F N K I F L P T I  
 1 M S N I T D P Q M W D F D D L . . . . . N F T G M P P A D E D Y S P C . M L E T E T L N K Y V V I I A  
 1 ME S D S F E D F W K G E D L S N Y S S T L P P F L D A A P C . E P E S L E I N K Y F V V I I  
 1 M N S F N Y T T P D . . . . . Y G H Y D D K D T L D L N T P V D K T S N T L R V P D I L A L V I  
 1 ME T . . . . . N S S L P T N I S G G T P A V S A G Y L F L D I I T Y L V  
 1 M T P T T T T A E L . . . . . T T E F D Y D E D A T P C V F T D V L N Q S K P V T L F L

C-CKR-I  
 HUMSTR  
 IL8rA  
 IL8rB  
 C5a  
 fMLP  
 hcmv-us28

41 Y S L V F V I G L V G N I L V V L V Q Y K R L K N M T S I Y L N L A I S D L L F L F T L P F W  
 45 Y S I I F L T G I V G N G L V I L V M G Y Q K K L R S M T D K Y R L H L S V A D L L F V I T L P F W  
 46 Y A L V F L L S L L G N S L V M L V I L Y S R V G R S V T D V Y L L N L A L A D L L F A L T L P I W  
 50 Y A L V F L L S L L G N S L V M L V I L Y S R V G R S V T D V Y L L N L A L A D L L F A L T L P I W  
 44 F A V V F L V G V L G N A L V V W V T A F . E A K R T I N A I W F L N L A V A D F L S C L A L P I L  
 33 F A V T F V L G V L G N G L V I W V A G F . R M T H T V T T I S Y L N L A V A D F C F T S T L P F F  
 40 Y G V V F L F G S I G N F L V I F T I T W R R I Q C S G D V Y F I N L A A A D L L F V C T L P L W

C-CKR-I  
 HUMSTR  
 IL8rA  
 IL8rB  
 C5a  
 fMLP  
 hcmv-us28

91 I D Y K L - K D D W V F G D A M C K I L S G F Y Y T G L Y S E I F F I I L L T I D R Y L A I V H A V  
 95 - A V D A - V A N W Y F G N F L C K A V H V I Y T V N L Y S S V L I L A F I S L D R Y L A I V H A T  
 96 A A S K V . - N G W I F G T F L C K V V S L L K E V N F Y S G I L L L A C I S V D R Y L A I V H A T  
 100 A A S K V . - N G W I F G T F L C K V V S L L K E V N F Y S G I L L L A C I S V D R Y L A I V H A T  
 93 F T S I V Q H H W P F G G A A C S I L P S L I L L N M Y A S I L L L A T I S A D R Y L A I V H A T  
 82 M V R K A M G G H W P F G W F L C K F V F T I V D I N L F G S V F L I A L I A L D R Y C V C V L H P V  
 90 M Q Y L L . . D H N S L A S V P C T L L T A C F Y V A M F A S L C F I T E I A L D R Y A I V Y . .

FIG. 1A

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C-CKR-1	140	F	A	L	R	A	R	T	V	T	F	G	V	I	T	S	I	I	W	A	L	A	I	L	A	S	M	P	G	L	Y	F	S	K	T	Q	W	E	F	T	H	H	T	..				
HUMSTR	143	N	S	Q	R	P	R	K	L	L	A	E	K	V	V	Y	V	G	V	W	I	P	A	L	L	T	I	P	D	F	I	F	A	N	V	S	E	A	D	D	R	Y	I	..				
IL8rA	144	R	T	L	T	Q	K	R	..	H	L	V	K	F	V	C	L	G	C	W	G	L	S	M	N	L	S	L	P	F	F	L	F	R	Q	A	Y	H	P	N	S	S	P	V	..			
IL8rB	148	R	T	L	T	Q	K	R	..	Y	L	V	K	F	I	C	L	S	I	W	G	L	S	L	L	A	L	P	V	L	L	F	R	R	T	V	Y	S	S	N	V	S	P	A	..			
C5a	153	W	C	Q	N	F	R	G	A	G	L	A	W	I	A	C	A	V	A	W	G	L	A	L	L	T	I	P	S	F	L	Y	R	V	V	R	E	E	Y	F	P	P	K	V	L			
fMLP	152	W	T	Q	N	H	R	T	V	S	L	A	K	V	I	I	G	P	W	V	M	A	L	L	T	L	P	V	I	..	R	V	T	T	V	P	G	K	T	G	T	V	A	C	T	F	N	F
hcmv-us28	156	..	M	R	Y	R	P	V	K	Q	A	C	L	F	S	I	F	W	I	F	A	V	I	I	A	I	P	H	F	M	V	V	T	K	K	D	N	Q	..									

\* C S L H F  
C D R F Y  
C Y E V L  
C Y E D M  
C G V D Y  
C T F N F  
C M T D Y

5

C-CKR-1	188	P	H	E	S	L	R	E	W	K	L	F	Q	..	..	A	L	K	L	N	L	F	G	L	V	L	P	L	L	V	M	I	I	C	Y	T	G	I	I	K	I	L	R								
HUMSTR	191	P	N	D	L	..	..	W	V	V	F	..	..	..	..	Q	F	Q	H	I	M	V	G	L	I	L	P	G	I	V	I	L	S	C	Y	C	I	I	I	S	K	L	S	H							
IL8rA	192	G	N	D	T	A	K	..	..	W	R	M	V	L	..	..	R	I	L	P	H	T	F	G	F	I	V	P	L	F	V	M	L	F	C	Y	G	F	T	L	R	T	L	F	K						
IL8rB	196	G	N	N	T	A	N	..	..	W	R	M	L	L	..	..	R	I	L	P	Q	S	F	G	F	I	V	P	L	I	M	L	F	C	Y	G	F	T	L	R	T	L	F	K							
C5a	193	S	H	D	K	R	R	E	R	A	V	A	..	..	..	..	I	V	R	L	V	L	G	F	L	W	P	L	L	T	L	T	I	C	Y	T	F	I	L	L	R	T	W	S							
fMLP	181	S	P	W	T	N	D	P	K	E	R	I	N	V	A	V	A	M	L	T	V	R	G	I	I	R	F	I	I	G	F	S	A	P	M	S	I	V	A	V	S	Y	G	L	I	A	T	K	I	H	K
hcmv-us28	178	D	Y	L	E	V	S	..	Y	P	I	I	L	..	..	..	N	V	E	L	M	L	G	A	F	E	V	I	P	L	S	V	I	S	Y	C	Y	Y	R	I	S	R	I	V	A	V					

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C-CKR-1	229	R	P	N	E	K	K	S	K	A	V	R	L	I	F	V	I	M	I	I	F	F	L	F	W	T	P	Y	N	L	T	I	I	S	V	F	Q	D	F	..												
HUMSTR	229	S	K	G	H	Q	K	R	K	A	L	K	T	T	V	I	L	I	L	A	F	F	F	A	C	W	L	P	Y	Y	I	G	I	S	I	D	S	F	I	L	L	E	I	I	K	Q	G	C	E	F	E	N
IL8rA	232	A	H	M	G	Q	K	H	R	A	M	R	V	I	F	A	V	V	L	I	F	L	C	W	L	P	Y	N	L	V	L	A	D	T	L	M	R	T	Q	V	I	I	Q	E	T	C	E	R	R	N		
IL8rB	236	A	H	M	G	Q	K	H	R	A	M	R	V	I	F	A	V	V	L	I	F	L	C	W	L	P	Y	N	L	V	L	A	D	T	L	M	R	T	Q	V	I	I	Q	E	T	C	E	R	R	N		
C5a	232	R	R	A	T	R	S	T	K	T	L	K	V	V	V	A	V	A	S	F	F	I	F	W	L	P	Y	Q	V	T	G	I	M	..	M	S	F	L	E	P	S	S	P	T	F	..						
fMLP	231	Q	G	L	I	K	S	S	R	P	L	R	V	L	S	F	V	A	A	A	F	F	L	C	W	S	P	Y	Q	V	V	A	L	I	A	T	V	R	I	R	E	L	L	Q	G	M	Y	..				
hcmv-us28	218	S	Q	S	R	H	K	G	R	I	V	R	V	L	I	A	V	V	L	V	F	I	I	F	W	L	P	Y	H	L	T	L	F	V	D	T	L	K	L	K	W	I	S	S	S	C	E	F	E	R		

FIG. 1B



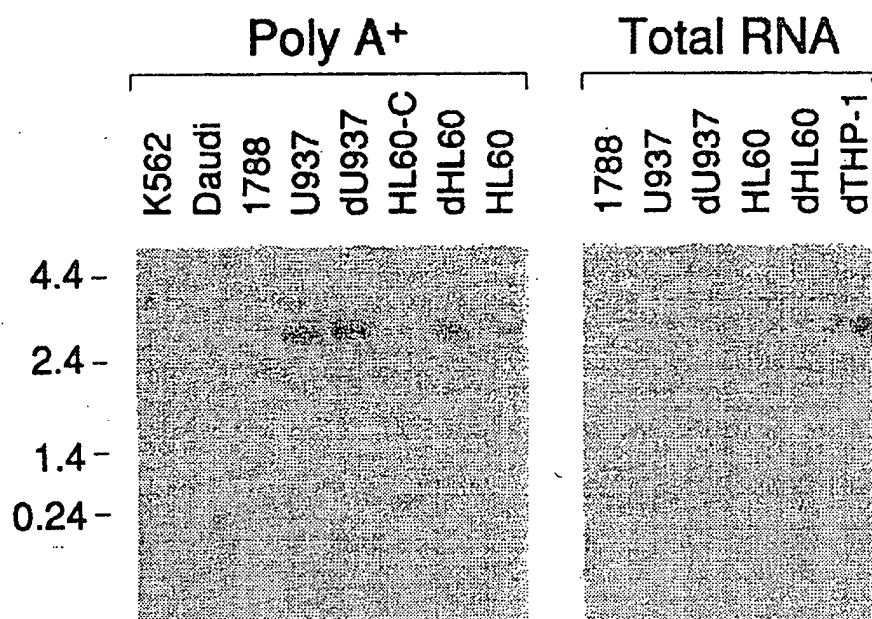


FIG. 2

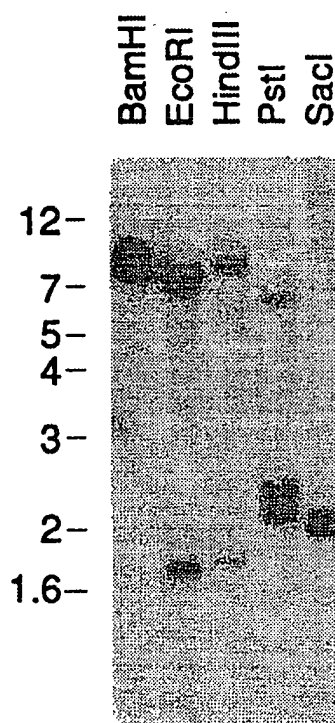


FIG. 3A



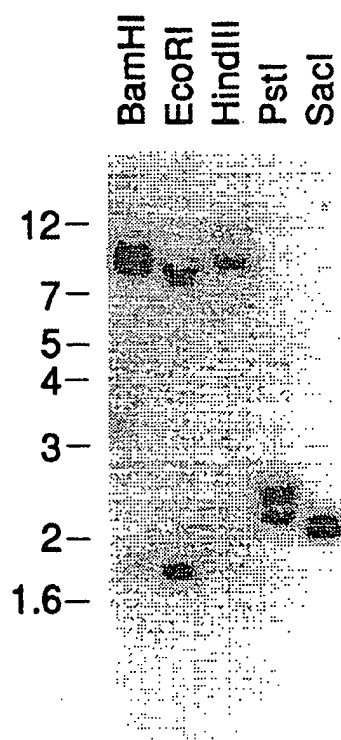
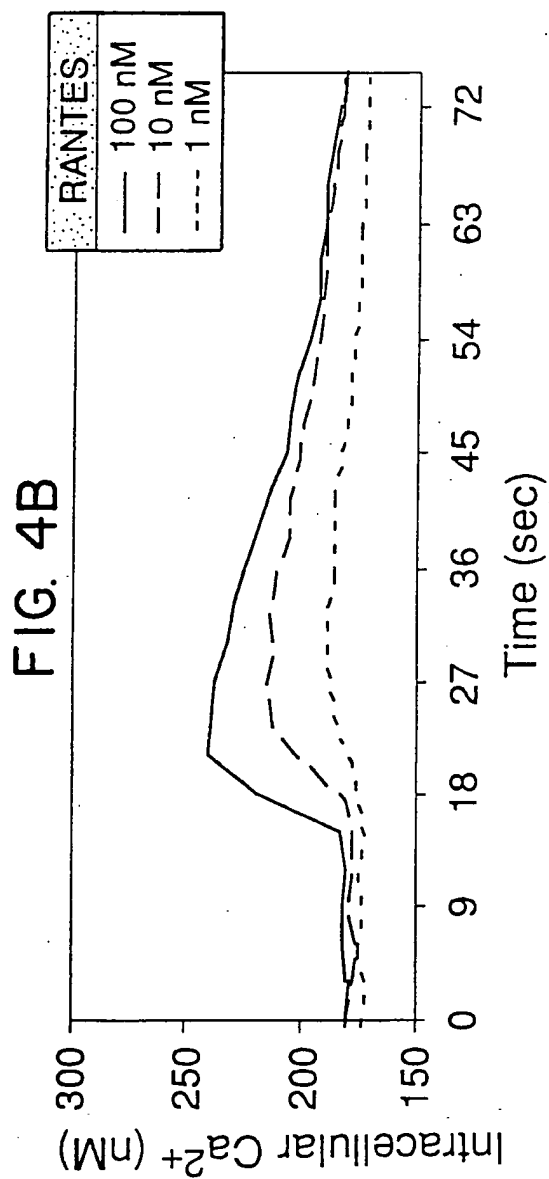
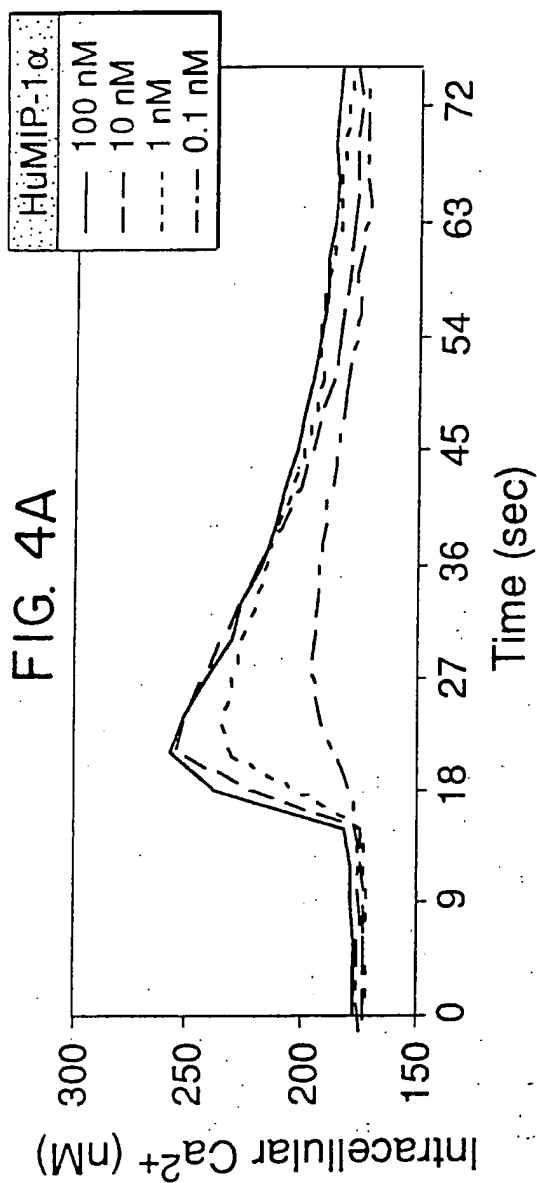
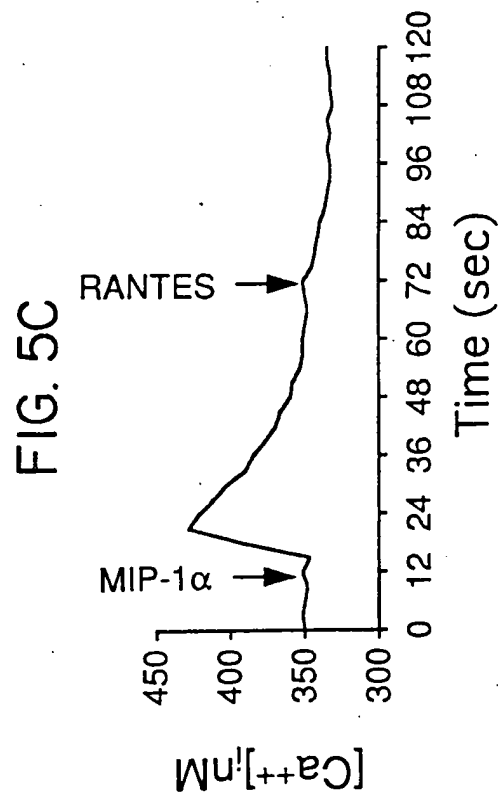
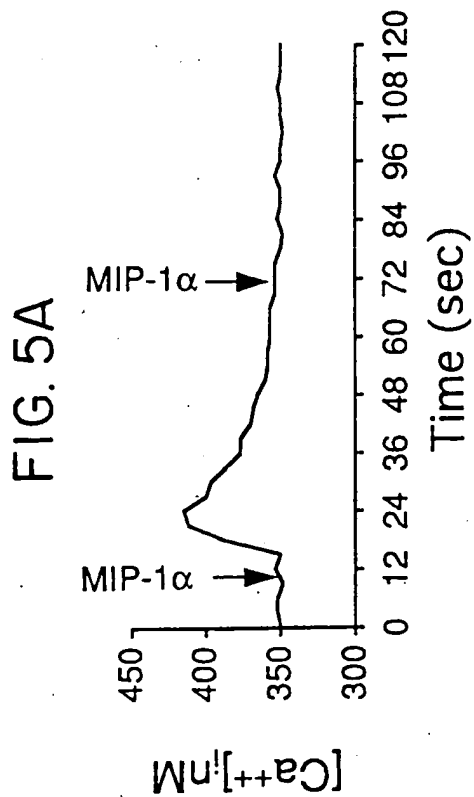
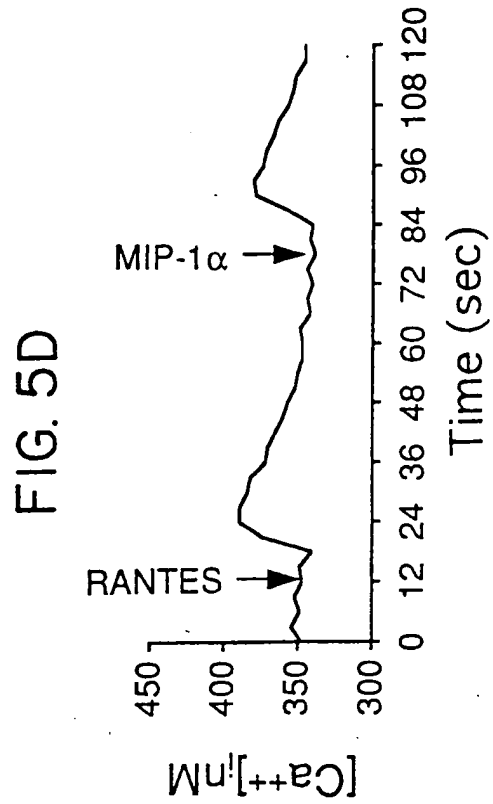
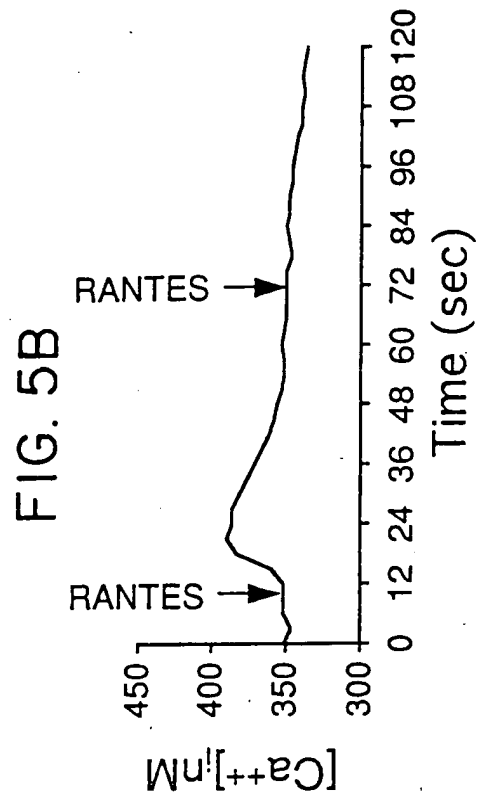


FIG. 3B

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FIG. 6A

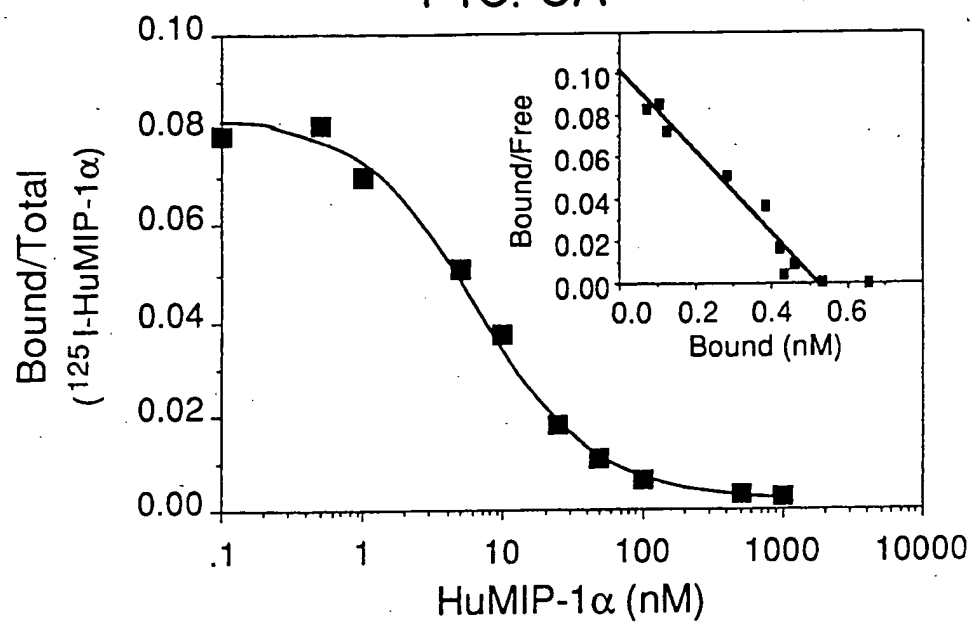
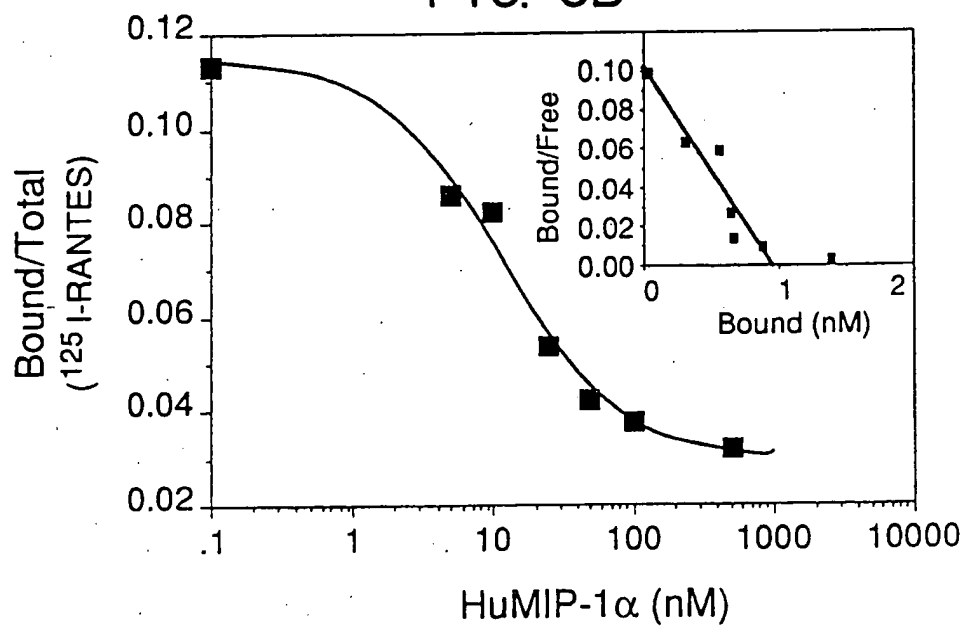
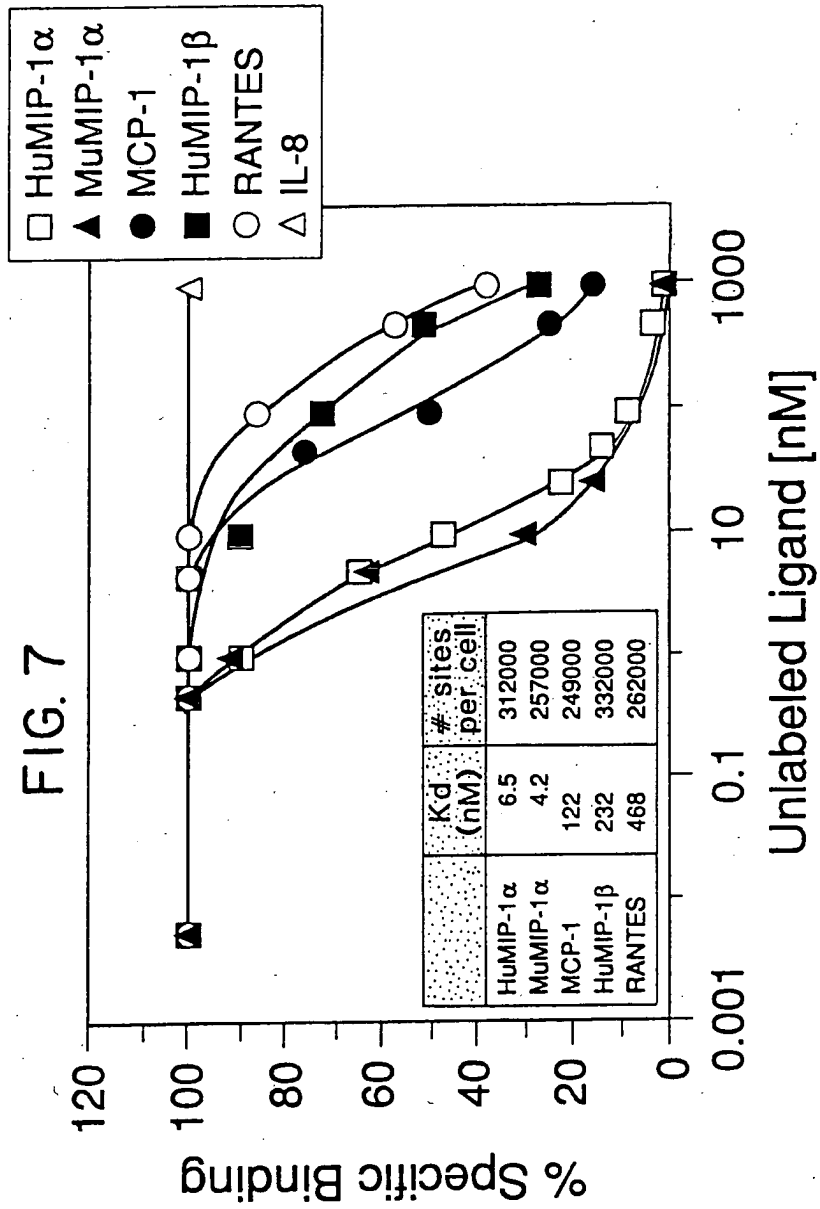


FIG. 6B



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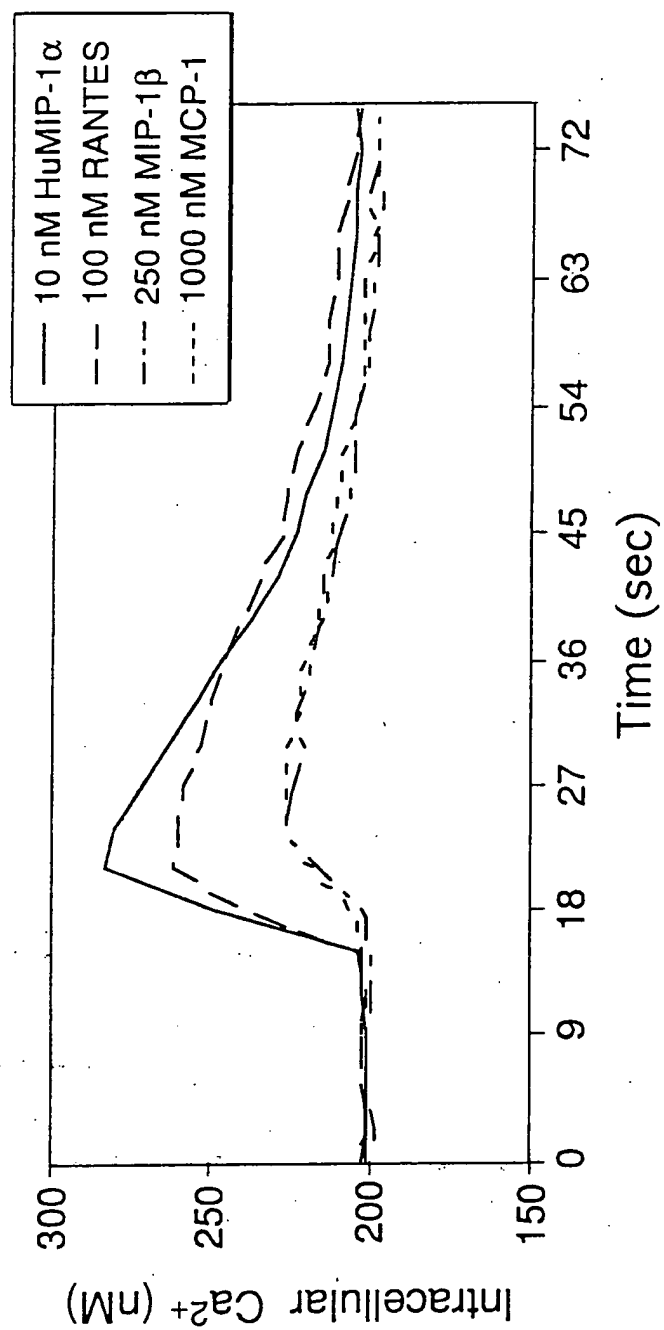


FIG. 8

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1	ATGGAACTC	CAAACACCAC	AGAGGACTAT	GACACGACCA	CAGAGTTTGA
	TACCTTTGAG	GTTTGTGGTG	TCTCCTGATA	CTGTGCTGGT	GTCTCAAAC
1	M E T P	N T T	E D Y	D T T T	E F D
	CTATGGGGAT	GCAACTCCGT	GCCAGAAGGT	GAACGAGAGG	GCCTTTGGGG
	GATACCCCTA	CGTTGAGGCA	CGGTCTTCCA	CTTGCTCTCC	CGGAAACCCC
	Y G D	A T P C	Q K V	N E R	A F G A
101	CCCAACTGCT	GCCCCCTCTG	TACTCCTTGG	TATTTGTCAT	TGGCCTGGTT
	GGGTTGACGA	GCCCCCAGAC	ATGAGGAACC	ATAAACAGTA	ACCGGACCAA
35	Q L L	P P L	Y S L V	F V I	G L V
	GGAAACATCC	TGGTGGTCCT	GGTCCTTGTG	CAATACAAGA	GGCTAAAAAA
	CCTTTGTAGG	ACCACCAGGA	CCAGGAACAC	GTTATGTTCT	CCGATTTTTT
	G N I L	V V L	V L V	Q Y K R	L K N
201	CATGACCAGC	ATCTACCTCC	TGAACCTGGC	CATTTCTGAC	CTGCTCTTCC
	GTACTGGTCG	TAGATGGAGG	ACTTGGACCG	GTAAAGACTG	GACGAGAAGG
68	M T S	I Y L L	N L A	I S D	L L F L
	TGTTACGCT	TCCCTTCTGG	ATCGACTACA	AGTTGAAGGA	TGACTGGGTT
	ACAAGTGCGA	AGGGAAGACC	TAGCTGATGT	TCAACTTCCT	ACTGACCCAA
	F T L	P F W	I D Y K	L K D	D W V
301	TTTGGTGATG	CCATGTGTAA	GATCCTCTCT	GGGTTTTATT	ACACAGGCTT
	AAACCACTAC	GGTACACATT	CTAGGAGAGA	CCCAAATAA	TGTGTCCGAA
101	F G D A	M C K	I L S	G F Y Y	T G L
	GTACAGCGAG	ATCTTTTTCA	TCATCCTGCT	GACGATTGAC	AGGTACCTGG
	CATGTCGCTC	TAGAAAAAGT	AGTAGGACGA	CTGCTAACTG	TCCATGGACC
	Y S E	I F F I	I L L	T I D	R Y L A
401	CCATCGTCCA	CGCCGTGTTT	GCCTTGCGGG	CACGGACCGT	CACTTTTGGT
	GGTAGCAGGT	GCGGCACAAA	CGGAACGCCC	GTGCCTGGCA	GTGAAAACCA
135	I V H	A V F	A L R A	R T V	T F G
	GTCATACCA	GCATCATCAT	TTGGGCCCTG	GCCATCTTGG	CTTCCATGCC
	CAGTAGTGGT	CGTAGTAGTA	AACCCGGGAC	CGGTAGAACC	GAAGGTACGG
	V I T S	I I I	W A L	A I L A	S M P
501	AGGCTTATAC	TTTTCCAAGA	CCCAATGGGA	ATTCACTCAC	CACACCTGCA
	TCCGAATATG	AAAAGGTTCT	GGGTTACCCT	TAAGTGAGTG	GTGTGGACGT
168	G L Y	F S K T	Q W E	F T H	H T C S
	GCCTTCACTT	TCCTCACGAA	AGCCTACGAG	AGTGGAAGCT	GTTTCAGGCT
	CGGAAAGTGAA	AGGAGTGCTT	TCGGATGCTC	TCACCTTCGA	CAAAGTCCGA
	L H F	P H E	S L R E	W K L	F Q A
601	CTGAAACTGA	ACCTCTTTGG	GCTGGTATTG	CCTTTGTTGG	TCATGATCAT
	GACTTTGACT	TGGAGAAACC	CGACCATAAC	GGAAACAACC	AGTACTAGTA
201	L K L N	L F G	L V L	P L L V	M I I
	CTGCTACACA	GGGATTATAA	AGATTCTGCT	AAGACGACCA	AATGAGAAGA
	GACGATGTGT	CCCTAATATT	TCTAAGACGA	TTCTGCTGGT	TTACTCTTCT
	C Y T	G I I K	I L L	R R P	N E K K

FIG. 9A

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701 AATCCAAAGC TGTCCGTTTG ATTTTGTGCA TCATGATCAT CTTTTTCTC
    TTAGGTTTCG ACAGGCAAAC TAAAAACAGT TGTACTAGTA GAAAAAAGAG
235 S K A V R L I F V I M I I F F L

    TTTTGGACCC CCTACAATTT GACTATACTT ATTTCTGTTT TCCAAGACTT
    AAAACCTGGG GGATGTTAAA CTGATATGAA TAAAGACAAA AGGTTCTGAA
    F W T P Y N L T I L I S V F Q D F

801 CCTGTTCAAC CATGAGTGTG AGCAGAGCAG ACATTTGGAC CTGGCTGTGC
    GGACAAGTGG GTAATCACAC TCGTCTCGTC TGTAACCTG GACCGACACG
268 L F T H E C E Q S R H L D L A V Q

    AAGTGACGGA GGTGATCGCC TACACGCACT GCTGTGTCAA CCCAGTGATC
    TTCACTGCCT CCACTAGCGG ATGTGCGTGA CGACACAGTT GGGTCACTAG
    V T E V I A Y T H C C V N P V I

901 TACGCCTTCG TTGGTGAGAG GTTCCGGAAG TACCTGCGGC AGTTGTTCCA
    ATGCGGAAGC AACCCTCTC CAAGGCCTTC ATGGACGCCG TCAACAAGGT
301 Y A F V G E R F R K Y L R Q L F H

    CAGGCGTGTG GCTGTGCACC TGGTTAAATG GCTCCCTTC CTCTCCGTGG
    GTCCGCACAC CGACACGTGG ACCAATTTAC CGAGGGGAAG GAGAGGCACC
    R R V A V H L V K W L P F L S V D

1001 ACAGGCTGGA GAGGGTCAGC TCCACATCTC CCTCCACAGG GGAGCATGAA
    TGTCCGACCT CTCCAGTCG AGGTGTAGAG GGAGGTGTCC CTCGTACTT
335 R L E R V S S T S P S T G E H E

    CTCTCTGCTG GGTTCTGACT CAGACCATAG GAGGCCAACC CAAAATAAGC
    GAGAGACGAC CCAAGACTGA GTCTGGTATC CTCCGGTTGG GTTTTATTCG
    L S A G F

1101 AGGCGTGACC TGCCAGGCAC ACTGACCAGC AGCCTGGCTC TCCAGCCAG
    TCCGCACTGG ACGGTCCGTG TGAATGGTGC TCGGACCGAG AGGGTCCGGT

    GTTCTGACTC TTGGCACAGC ATGGAGTCCG CCTCTTGGAT AGAGAGGAAT
    CAAGACTGAG AACCGTGTG TACCTCAGGC GGAGAACCTA TCTCTCCTTA

1201 GTAATGGTGG CCTGGGGCTT CTGAGGCTTC TGGGCTTGAG TCTTTTCCAT
    CATTACCACC GGACCCCGAA GACTCCGAAG ACCCGAACTC AGAAAAGGTA

    GAACTTCTCC CCTGGTAGAA AAGAAGATGA ATGAGCAAAA CCAATATTC
    CTTGAAGAGG GGACCATCTT TTCTTCTACT TACTCGTTTT GTTTTATAAG

1301 CAGAGACTGG GACTAAGTGT ACCAGAGAAG GGCTTGGACT CAAGCAAGAT
    GTCTCTGACC CTGATTCACA TGGTCTCTTC CCGAACCTGA GTTCGTTCTA

    TTCAGATTTG TGACCATTAG CATTTGTCAA CAAAGTCACC CACTTCCCAC
    AAGTCTAAAC ACTGGTAATC GTAAACAGTT GTTTCAGTGG GTGAAGGGTG

1401 TATTGCTTGC ACAAACCAAT TAAACCCAGT AGTGGTGACT GTGGGCTCCA
    ATAACGAACG TGTTTGTTA ATTTGGGTCA TCACCACTGA CACCCGAGGT

    TTCAAAGTGA GCTCCTAAGC CATGGGAGAC ACTGATGTAT GAGGA
    AAGTTTCACT CGAGGATTCG GTACCCTCTG TGACTACATA CTCCT

```

FIG. 9B

SUBSTITUTE SHEET (RULE 26)



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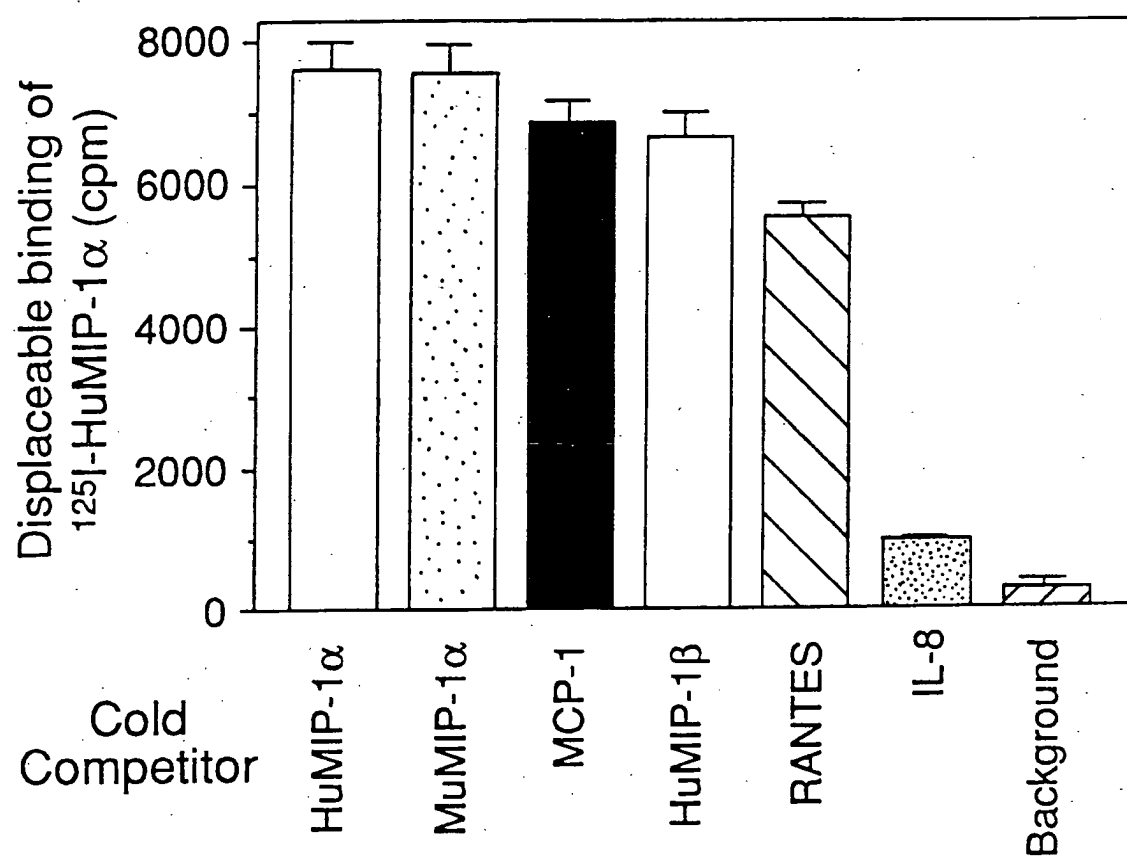


FIG. 10

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 93/10672

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/12 C07K13/00 C12P21/08 C12N5/10 C12N15/62  
A61K37/02 A61K39/395 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12N C07K C12P C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	CELL vol. 72 , 12 February 1993 , CAMBRIDGE, NA US pages 415 - 425 NEOTE K;DIGREGORIO D;MAK JY;HORUK R;SCHALL TJ 'Molecular cloning, functional expression, and signaling' see the whole document ---	1-27
Y	WO,A,92 17497 (GENENTECH, INC. , US) 15 October 1992 see claims 8,15 ---	22-26
Y	WO,A,92 18641 (THE TRUSTEES OF BOSTON UNIVERSITY, US) 29 October 1992 see page 11, line 8 - page 12, line 12 -----	22-26

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

16 February 1994

Date of mailing of the international search report

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 93/10672

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9217497	15-10-92	EP-A- 0577752	12-01-94
WO-A-9218641	29-10-92	CA-A- 2107682	11-10-92
		EP-A- 0579739	26-01-94